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(54) Title: A METHOD OF CONTROLLING NEMATODE INFECTION IN ANIMALS AND A COMPOSITION THEREFOR

(57) Abstract

The number of infective, animal parasitic nematodes in an environment inhabited by animals susceptible to nematode infection may be reduced by administering to the animals a fungal material (mycelium or spores) of a nematode-destroying (especially predatory) fungus so as to provide an adequate nematode-controlling amount of the fungal material in the animal faeces. In order to ensure a sufficiently high viability of the fungal material during passage through the gastrointestinal tract of the animal, the composition is formulated in such a way that the fungal material is protected against gastrointestinal fluids, for instance by providing a suitable coating on the fungal material or embedding it in a convenient matrix.

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A method of controlling nematode infection in animals and a composition therefor.

FIELD OF INVENTION

The present invention relates to a method of reducing the number of infective, animal parasitic nematode larvae in an environment of animals so as to reduce the transmission of nematode infection to the animals inhabiting said environment; a composition for controlling animal parasitic nematodes; and a process for preparing a fungal material of a nematode-destroying fungus.

TECHNICAL BACKGROUND

Nematode-destroying fungi are an extremely common group of more than 150 microfungi (Barron, 1977 (1)). Most species are found within the order *Moniliales* (*Hyphomycetes*) of the class *Fungi imperfecti*, while some belong to the order *Zoopagales* of the class *Phycomycetes*. A key by Cooke & Godfrey (1964) (2) lists more than 90 species. Today there are several reviews on aspects of the biology of nematode-destroying fungi (Drechsler, 1941 (3); Soprunov, 1958 (4); Duddington, 1962 (5); Pramer, 1964 (6); Sayre, 1971 (7); Barron, 1977; Peloille, 1981 (8); and Mankau, 1981 (9)).

Nematode-destroying fungi may be divided into three groups according to their mode of action: a predatory, an ovoparasitic and an endoparasitic group. Protozoans, rotifers and especially nematodes are organisms that may be trapped by the group of predatory fungi. To trap nematodes, the predatory fungi develop different kinds of capturing organs like constricting or non-constricting rings, sticky hyphae, knobs, branches or, for instance in the case of *Arthrobotrys oligospora*, three-dimensional sticky networks which are the most common type of trapping organ. As is the case with many predatory fungi, the presence of nematodes is necessary for the induction of traps in *A. oligospora* (Nordbring-Hertz, 1977 (11)). Studies by and Jansson & Nordbring-Hertz, 1979 (13), suggest that the living mycelium of *A. oligospora* also attracts nematodes. Once the nematode is trapped, its cuticle is penetrated and an infection bulb is formed from which

trophic hyphae grow into the body of the nematode and absorb its contents (Shepherd, 1955 (14)). Studies by Olthof & Estey, 1963 (15), seem to suggest that when the nematode cuticle is penetrated by *A. oligospora*, the trophic hyphae give off substances which are toxic to nematodes.

The group of nematode-destroying fungi is widely distributed and may be isolated from leaf mould, rotting wood, partially decayed plant residues, moss and dung (Duddington, 1951 (16)). *Arthrobotrys oligospora*, one of the most widely distributed predatory fungi, is the most common predatory fungus in agricultural soils in a temperate climate (Shepherd, 1961 (17)), and it may also be isolated from cattle dung (Juniper, 1957 (18)). In fact, Zopf (1888) (19) described it as a dung-inhabiting fungus.

Growth and trap formation of predatory fungi is necessary for nematode destruction. In general, predatory fungi are capable of saprophytic development but they are poor competitors in many soils which often contain factors fungistatic to conidia and perhaps other germinating units (Cooke, 1968 (20)). Conidia in such soils are lysed or capturing organs are formed directly from the conidia (Mankau, 1962 (21)). Traps created directly from conidia may represent an adaption of the predatory fungi to survive in highly antagonistic soils using only nematodes as feed.

Predatory fungi occur in nature but it has proved very difficult to alter the environment of the soil to favour predatory fungi by the admixture of organic matter (defined as a residue or waste of biological processes or decomposing matter of biological origin, e.g. compost or manure) or fungal material.

In nutrient-poor soils, predatory fungi are more or less inactive until a fresh energy source such as organic matter is added as this stimulates bacterial growth and hence nematode multiplication. On the other hand, when large amounts of organic matter are added to the soil, the activity of predatory fungi may decrease as a result of an increased intensity of competition from other soil fungi.

When predatory fungi are applied directly to soil, some species appear to be able to compete successfully with the soil microflora at higher inoculum levels (Cooke, 1963 (23)). However, there are great difficulties in establishing profitable productions of the sufficient amounts of fungal material necessary for predatory fungi to be as effective as chemical agents used in modern agricultural production systems. It may therefore be expected that in many cases biological control agents will act less quickly and directly than chemicals.

Biological control of plant parasitic nematodes by adding organic matter, cultures of nematode-destroying fungi or both to soil has been successful in a few cases, as indicated in the examples summarized below, but most efforts have been inconclusive or negative (Cooke, 1968 (20)).

Most investigations of the effect of nematode-destroying fungi have been concerned with plant parasitic nematodes in soil (Kerry, 1984 (24)). Some of these investigations have demonstrated an already existing biological control of plant parasitic nematodes in the field by naturally occurring nematode-destroying fungi (Stirling et al., 1978 (25) and Kerry et al., 1980 (26)).

Work on the control of plant parasitic nematodes was initiated by Linford (1937) (27) in Hawaii. In this experiment, the incorporation of fresh plant material in naturally infested soil resulted in a reduction of the number of infective larvae of *Heterodera marioni*, perhaps as a result of increased activity of nematode-destroying fungi present in the plant material.

Admixture of the predatory fungus itself to soil as biological control of plant parasitic nematodes has been studied extensively in France, where two commercial products have been produced.

Cayrol et al. (1978) (28) developed a product based on a horse manure isolate of *Arthrobotrys robusta* cultivated on rye grains (Royal 300°). The fungal product was reported to protect the commercial mushroom, *Agaricus bisporus*, from attack by the mycophagous nematode *Ditylenchus myceliophagus*. *A. robusta* had no adverse effect on the development of the mushroom mycelium and grew rapidly in mushroom

compost. In mushroom cultures seeded with 1% *A. robusta* product, there was a 40% reduction of the *D. myceliophagus* population, and the mushroom harvest was increased by more than 20%.

Cayrol & Frankowski (1979) (29) developed another product of *Arthrobotrys irregularis* grown commercially on rye grains (Royal 350®). In field trials, *A. irregularis* was applied to the soil at least one month in advance of planting at a rate of 140 g/m^2. The results showed a good protection of tomato plants against the root-knot nematode *Meloidogyne*.

In the field of medical helminthology, Soprunov (1958) (4) and his staff carried out extensive laboratory and field trials of predatory fungi in Russia. It was found that a number of isolated predatory fungi were able to destroy hookworm larvae such as *Ancylostoma duodenale* and *Necator americanus* pathogenic to human beings. In the period from 1954 to 1956, predatory fungi were annually spread at a rate of 100-150 g of fungal spore powder per m^2 over the soil in a coal mine where hookworm infection was a serious problem among the miners. A total of 400 kg of spore powder was spread in the mine over a period of 3 years. In 1953 *Trichothecium globosporum, T. pravicovi, Arthrobotrys dolioformis, A. kirghizia, A. arthrobotryoides* and *A. superba* grown on chopped corn were used to produce a total of 90 kg of spore powder. Later, from 1954 onwards, about 310 kg of spore powder were prepared using *Arthrobotrys oligospora* and *A. dolioformis* grown on oatmeal medium or chopped corn cobs. Following the introduction of predatory fungi into the coal mine, the incidence of hookworm disease among the miners diminished rapidly, presumably because the fungi killed the hookworm larvae. The absence of any complaints which could be attributed to mycosis suggests that predatory fungi are not pathogenic to human beings. A product was made from the two most promising predatory fungi, *Arthrobotrys oligospora* and *A. dolioformis*, cultured on chopped corn and oatmeal (Soprunov & Tendetnik, 1960 (30)).

Only few investigations of the possibility of using predatory fungi for controlling animal parasitic nematodes have been made.

A number of preliminary laboratory experiments have been carried out for the last fifty years. In France, Descazeaux (1939) (31) found that *Arthrobotrys oligospora* was able to trap and consume parasitic trichostrongyle nematode larvae from cattle and sheep. Deschiens (1939) (32) reported that on agar the trapping network of *A. oligospora* was formed within 36 hours after contact with strongyle parasite larvae from cattle. Later laboratory experiments have shown that *A. oligospora* may destroy parasitic strongyle nematode larvae of the donkey (Soprunov, 1958 (4)), infective larvae of *Trichostrongylus axei* and *Ostertagia ostertagi* of cattle (Pandey, 1973 (33)), *Haemonchus contortus* of sheep (Virat & Peloille,1977 (34)) and *Cooperia* spp. of cattle (Grønvold et al., 1985 (35)). In an extensive study, Nansen et al. (1987) (36) showed that *A. oligospora* is capable of destroying a number of infective parasitic nematode larvae of cattle (*Ostertagia ostertagi*, *Cooperia oncophora*, *Dictyocaulus viviparus*), sheep (*Cooperia curticei*, *Haemonchus contortus*), pigs (*Oesophagostomum dentatum*, *Oe. quadrispinulatum*) and horses (*Cyatostoma* spp.). Comparisons made among the first, the second and the third and infective larval stages of *Cooperia oncophora* showed no difference in the ability of the different stages to induce networks in *A. oligospora* (Nansen et al., 1986 (37)). All stages were trapped with the same efficiency.

Only a few field experiments on the practical use of predatory fungi have been performed. Roubaud & Deschiens (1941) (38) carried out an experiment in which two small plots of pasture (5 x 5 m) infected with *Strongyloides papillosus* and *Bunostomum* spp. were each grazed by two ten-month-old lambs. One plot had been treated with spore powder of *Arthrobotrys oligospora*, *Dactylella bembicodes* and *Dactylaria ellipsospora*. After grazing for five weeks, the lambs were examined. It was found that the animals which had grazed in the treated plot had a significantly lower parasite burden than the lambs from the control plot. It is difficult, however, to base any final conclusions on the effectiveness of predatory fungi on this experiment as only two lambs were used in each group. Deschiens (1939) (31) found that on meadow grassland *A. oligospora* was able to grow and sporulate on both soil surface and on parts of the vegetation close to the ground. Application of the fungus did not harm the vegetation.

In another small field experiment, the predatory fungus *Arthrobotrys oligospora* was added directly to cow pats containing eggs of *Cooperia oncophora*, a parasitic nematode of cattle (Grønvold et al., 1987 (39)). The cow pats were placed in a parasite-free pasture. This study was planned to achieve a closer contact between developing parasite larvae and the predatory fungi than could be obtained by spreading fungal material evenly over the pasture. The results showed that the number of infective *C. oncophora* larvae isolated from the cow pats as well as from the surrounding herbage were subject to an approximately ten-fold reduction compared with the number of larvae found in fungus-free control pats and herbage surrounding these. Laboratory experiments have shown that admixture of 2500 conidia of *A. oligospora* to cattle faeces results in a 99% reduction in infective *Cooperia* larvae in faecal cultures (Grønvold et al., 1985 (35)).

It has been attempted to obtain admixture of predatory fungi with animal faeces by adding predatory fungal material to the feed of nematode-infected animals provided that the fungi can pass the alimentary tract in a viable state. However, Descazeaux & Capelle (1939) (40) found that conidia of *Arthrobotrys oligospora* and *Dactylella bembicodes* administered orally to horses and guinea pigs were killed in the alimentary tract.

On the other hand, Soprunov (1958) (4) tested the viability of *Arthrobotrys oligospora* after passage through the intestinal tract of a donkey. *A. oligospora* was cultivated on chopped corn at temperatures between 25°C and 30°C and high humidiy for about one month. The final product contained 1.5-2 million conidia per gramme of spore powder. For five days, the donkey's fodder was supplemented with 150 g of spore powder. From the 2nd to the 9th day, *A. oligospora* was detected in the faeces of the donkey which was infected with strongyle nematode parasites. Over the first 6 days after ingestion of the spore powder, the strongyle larval population compared with that found in faecal samples taken prior to the ingestion of spores had been reduced by 80-90%. The results may suggest that *A. oligospora* is able to grow and trap nematodes after passage through the alimentary tract of a donkey. There is no indication of the proportion of surviving *A.*

oligospora after passage through the gastrointestinal tract. Ingestion of such large quantities of conidia was not found to harm the animal.

Gruner et al. (1985) (41) cultivated *Dactylaria candida*, *Candelabrella (Arthrobotrys) musiformis* and *Arthrobotrys tortor* on cooked millet seeds. This culture was administered to lambs at a rate of 100 g per 6 kg of body weight. The fungi passed the alimentary tract within the next 2 or 3 days. At that time, the faeces contained viable fungi and the results led the authors to suggest that the fungi were able to prey on the nematode parasites *Teladorsagia (Ostertagia) circumcincta* and *Haemonchus contortus*. No data are presented of the proportion of viable fungi compared to the initial amount administered.

The available literature on the control of animal parasitic nematodes by means of predatory fungi is inconclusive in several respects. In particular, there are no quantitative studies of the viability of the fungus after gastrointestinal passage and the data presented by Soprunov and Gruner are based on so little experimental material (one animal and four animals, respectively) that the results obtained from the experiments may not be reproducible. It may well be that other experiments would show that the fungus is in fact not viable (cf. Descazeaux & Capelle, *op.cit.* (40)) or shows a very low viability count, and that viability might, *inter alia*, depend on the particular fungal species or strain employed, or on the individual animal to which the fungus is administered. Also, there is no evidence of a persistent effect in a natural epidemiological situation. At any rate, it would appear from the publications summarized above that very large quantities of the fungus need to be administered in order to ensure an adequate viability of the fungus for the purpose of nematode control.

On the other hand, there is a need for a method of biological long-term control of nematodes as resistance of nematodes to chemical anthelmintics has become an increasingly severe problem in the field of animal husbandry. Consequently, it would be desirable to devise a reproducible method of combating animal parasitic nematodes by means of a nematode-destroying fungus in which a composition which is substantially uniform with respect to the initial content of fungal material

and the viability thereof after gastrointestinal passage is administered to the animals.

SUMMARY OF THE INVENTION

One object of the present invention is therefore to provide a method of controlling animal parasitic nematodes wherein a fungal material of a nematode-destroying fungus is provided in animal faeces by gastrointestinal passage, the fungal material retaining an adequate degree of viability after the gastrointestinal passage.

Accordingly, the present invention relates to a method of reducing the number of infective, animal parasitic nematodes in an environment of animals so as to reduce the transmission of nematode infection to animals inhabiting said environment, the method comprising administering to an animal a composition comprising a fungal material of a nematode-destroying fungus in order to provide an adequate nematode-controlling amount of the fungal material in the animal faeces, the composition being formulated in such a way that an adequate nematode-controlling proportion of the fungal material remains viable after passage through the gastrointestinal tract of the animal to which the composition is administered.

The nematodes which the method of the invention aims to control may be any of the nematode species known to infect animals, including human beings, and to cause more or less severe damage in the host which they infect. Examples of nematodes to be controlled by the method of the invention are *Ostertagia* spp., *Trichostrongylus* spp., *Haemonchus* spp., *Dictyocaulus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Cyathostoma* spp., *Strongyloides* spp., *Strongylus* spp., *Bunostomum* spp., *Ancylostoma* spp. and *Necator* spp. Dependent on the type of nematode to be controlled, the composition to be administered may include, as the fungal material of the nematode-destroying fungus, a fungal material from a predatory fungus (which is capable of capturing nematode larvae), an endoparasitic fungus (which is capable of establishing itself in nematode larvae) or an ovoparasitic fungus (which is capable of establishing itself in helminth eggs). The nematode-destroying fungus is suitably a predatory fungus.

The trapping devices of predatory fungi may take various forms as indicated above. The formation of adhesive networks is stimulated by the presence of nematode larvae which are trapped in loops formed in the network. In contact with the larvae, the loops secrete an adhesive substance to hold back the larvae. A similar trapping mechanism is found in another type of trapping device consisting of adhesive branches which, however, does not form loops. After attachment an outgrowth of the fungus penetrates the larval cuticle and swells to a bulb-like growth inside the larva. From this bulb trophic hyphae grow which consume the larval body contents so that, finally, only the cuticle is left. When the trapping devices consist of adhesive knobs, the larva is attached to several of these knobs some of which produce trophic hyphae consuming the larval contents and others of which presumably give off a toxic substance. A further type of trapping devices consists of constricting rings where a branch forms a closed ring of three curved cells which swell up when a larva enters the ring so that the larva is held tightly and cannot escape. The trophic hyphae then penetrate the cuticle and consume the body contents of the larva.

In the present context, the term "transmission" is intended to mean that the nematode infection is transferred from infected animals to non-infected animals present in the same environment.

The term "fungal material" is understood to mean a material which comprises all parts of a fungus, including the mycelium (hyphae) and all types of spores, e.g. conidia and chlamydospores, or a mixture thereof. It has surprisingly been found that the mycelium may also be employed as the material from which the active, that is nematode-destroying, fungus may be developed at the site where such fungi are usually active (i.e. in faeces).

The term "adequate nematode-controlling amount" is intended to indicate that the amount of fungal material present in the animal faeces is sufficient to bring about a significant reduction of the number of infective nematodes in the environment, for instance an enclosure, of an animal to which the composition containing the fungal material is administered, and a consequent reduction of the transmission of nematode infections to animals present in the environment treated with

the fungal material. Such an adequate amount of the fungal material may presumably be provided by administering large quantities thereof to the animals which are to be protected from nematode infection, so as to ensure the survival of an adequate proportion of the material even in unprotected form in the alimentary tract of the animal in question, but this is a less desirable procedure for two reasons, namely the problems associated with the production of large quantities of fungal material and the possible difficulties in making the animal ingest the requisite large amount of fungal material. It is therefore preferred to provide the fungal material in protected form so that an improved viability of the fungal material is ensured, requiring the administration of, presumably, far smaller quantities of the fungal material, thereby improving the reproducibility of the method of the invention. In this context, the term "reproducibility" is intended to indicate that it may be possible to make substantially all animals which, for instance, belong to the same stock ingest substantially equal amounts of the fungal material per kg body weight.

The protection of the fungal material may also serve to improve the storage stability of the fungal material in that it may further protect it from, for instance, absorption of water and exposure to oxygen which may result in a deteriorated long-term viability of the fungal material. This would tend not to be the case with corresponding unprotected fungal material, and in order to ensure an adequate supply of this viable unprotected material, it would be necessary to produce it in the immediate vicinity of the site of use and use it virtually immediately upon reaching a sufficient density of the production culture. The protection of the fungal material to be employed in the method of the invention therefore contributes to lowering the production costs of the fungal material by permitting a more rationalized production which, together with the decrease of the amount of fungal material necessary to administer to obtain a sufficient nematode control when the fungal material is in protected form, makes it less expensive to employ the method of the invention for nematode control than utilizing a corresponding method which does not make use of a protected fungal material.

DETAILED DISCLOSURE OF THE INVENTION

The choice of a particular species of nematode-destroying fungus to be incorporated in the composition employed in the method of the invention will of course in each case depend on the specific type of nematode to be combated, i.e. whether it is susceptible to an endoparasitic, ovoparasitic or predatory fungus. Many nematodes which constitute a serious problem in the field of animal husbandry may, however, be controlled by means of predatory fungi, and the composition to be administered to animals in accordance with the principles of the invention therefore advantageously contains a fungal material of a predatory fungus.

It has previously been shown (cf. Drechsler, 1941 (3), Soprunov, 1958 (4), Nansen et al., 1987 (36)) that as predatory fungi show little species specificity within the capacity of their predatory organs (the physical limitations of their predatory organs being the decisive factor), the choice of a particular predatory fungus to be employed in the method of the invention is presumably not critical in this respect. The fungus to be used may therefore be selected according to other criteria such as its fermentation properties (determining whether it is feasible to produce it on a large scale, and hence the production costs), growth properties at the site of use (it should preferably exhibit fast growth so as to be able to compete successfully with other organisms in the animal faeces) and optimum growth temperature. It is assumed, however, that for most practical applications, a predatory fungus belonging to an *Arthrobotrys* spp. or a *Dactylaria* spp. may be employed. Examples of species of predatory fungi to be included in the composition are *Arthrobotrys oligospora*, *Arthrobotrys tortor*, *Arthrobotrys musiformis*, *Arthrobotrys conoides*, *Arthrobothrys superba*, *Arthrobotrys arthrobotryoides* or *Dactylaria candida*. The composition may further include mixtures of any of these fungal species with, for instance, different temperature optima or other growth properties in order to provide a composition which may be employed under widely varying climatic conditions, e.g. the temperature, or the structure and composition of the soil.

The animals to which the composition is administered according to the method of the invention are usually domestic animals, that is, animals which are bred for commercial purposes, and are most often young animals since young animals are particularly susceptible to nematode infections. Furthermore, the animals are typically untethered animals as these are more vulnerable to nematode infection than those which are kept in separate enclosures.

The animals to which the composition comprising the fungal material is to be administered according to the invention may be pigs, in which case the environment where nematode control is to take place may be a pigsty or a pasture. In order to obtain a satisfactory nematode control where pigs are concerned, the composition containing the fungal material is suitably administered at least once every two days, preferably at least once a day, for at least one month, preferably for at least two months, during a period where contamination of the enviroment by nematodes is critical. The expression "period where contamination of the environment by nematodes is critical" should, in the present context, be understood to mean a period where the potential for development of infective nematode larvae and consequently the risk of transmitting infective material from one animal to the other is particularly high.

An estimated daily dosage of active fungal material is in the range of 1-10 mg (dry weight) of the fungal material per kg of faeces.

A group of animals to which the method of the invention may advantageously be applied is domestic fowl. The frequency with which the composition containing the fungal material is administered is typically similar to that applicable to pigs.

Another group of animals to which the composition containing the fungal material may be administered is carnivores, for instance fur animals such as mink or fox, or pets such as dogs or cats. The frequency with which the composition containing the fungal material is administered as well as the estimated daily dosage thereof is typically similar to that applicable to pigs.

The method of the present invention may advantageously be practised on herbivores.

A herbivore which is of particular interest in connection with the method of the present invention is the horse, as the problem of nematode resistance to conventional chemical anthelmintics has become increasingly serious with respect to horses in recent years. In the case of horses, the composition may be administered both to horses kept in a stable and kept in a pasture, but is, perhaps, of the greatest relevance to horses more or less permanently pastured or at the time of the year when the animals are turned to grass since horses are usually kept in separate enclosures in a stable where the risk of contamination is less grave. Where horses are concerned, the composition should advantageously be administered at least once every two days, preferably at least once a day, for at least two months, during a period where contamination of the environment by nematodes (in this case, typically when the animals are turned to grass) is critical. An estimated daily dosage of active fungal material is in the range of 1-10 mg (dry weight) of the fungal material per kg of faeces.

Other herbivores of particular interest in this connection are ruminants, e.g. cattle, sheep, deer or goats. In the case of ruminants, too, it is of the greatest importance to administer the composition to animals kept in a pasture where the intensity of nematode infection is particularly high. The composition should preferably be administered to the ruminants at least once (the frequency of administration depending on how the composition to be administered is formulated), at a time of the year when contamination of the environment by nematodes is critical for the build-up of high infectivity in the environment. This typically occurs at a time of the year when the animals are turned to grass or when young animals which have never been pastured before are pastured together with older animals some of which will most likely be infected by nematodes. The composition is suitably administered at least once over a period of at least 1 month, although the frequency of administration will vary widely depending on the type of formulation administered.

As explained above, it is preferred that the fungal material employed in the method of the invention is provided in protected form so as to obtain a controlled release of the fungal material. The release of the fungal material should preferably be adapted to ensure the highest possible survival rate of the fungal material which means that the release will usually be directed to occur in the distal part of the intestines or in the faeces so as to allow growth of the fungus in the faeces. The controlled release may be achieved by means of a coating or a matrix with specific solubility or disintegration characteristics, and the composition to be administered according to the invention may therefore be in the form of a matrix tablet or pill in which the fungal material is embedded, a coated tablet or capsule containing the fungal material or coated granules of the fungal material which is released on disintegration of the coating. When the animals to which the composition is to be administered are ruminants, the composition may advantageously be in the form of a bolus in which the fungal material is embedded and from which it is slowly and continuously released. A bolus preparation has the advantage that it need only be administered once or twice during the critical period, whereas for any of the other dosage forms, the composition will have to be administered with the same frequency as applies to any of the non-ruminants mentioned above.

As the fungi do not act immediately in destroying nematodes, that is, they require a certain period of time to develop, it may be necessary or at least convenient to combine the biologcial nematode-controlling agent with a chemical agent (cf. also Sayre, 1971 (7)).

Concurrently with administering the composition containing the fungal material, it may therefore often be an advantage to administer a chemical antiparasitic agent such as an anthelmintic which acts immediately to reduce the initial parasite "burden" (i.e. the number of parasites with an infective potential present in an animal) in each animal to which the composition is administered, thereby reducing the overall initial parasite population of the environment in which the animals are kept which may require a smaller amount of fungal material to be administered in order to provide a continuous reduction of the number of infective nematodes over a longer period of time. It

is, however, expected that the amounts of such a chemical anthelmintic will be far lower than if the chemical was administered alone, so that problems concerning possible residues of the drug in the meat or milk of the animals or concerning possible toxic effects of the drug in the animals are not likely to occur. The antiparasitic agent may also be one which is effective against other parasites than those controlled by the fungus incorporated in the composition.

The present invention further relates to a composition for controlling animal parasitic nematodes, which composition comprises a fungal material of a nematode-destroying fungus and an excipient which significantly improves the viability of the fungal material in the gastrointestinal tract of an animal to which the composition is administered. The fungal material included in the composition may be derived from any of the sources (genera or species of fungus) indicated above, and may comprise mycelium or spores or a mixture thereof.

The composition may be formulated in accordance with any of the known methods of formulating veterinary preparations. To effect an optimum survival rate of the fungal material and hence an optimum amount of fungal material at the site of activity, i.e. the animal faeces, it is preferred to provide a form of the composition that ensures protection of the fungal material in the alimentary tract of the animal to which the composition is administered, as explained in detail above. Such protection may be obtained by embedding the fungal material in a matrix which may be formulated as tablets or pills, the matrix material being insoluble in a gastrointestinal environment or soluble or erodible in the large intestine. The matrix formulation is optionally coated with a suitable coating such as one of those indicated below. The matrix material may suitably be selected from a natural or synthetic wax, a plastic, a polymer, a polysaccharide, such as an alginate, a dextrin, a starch, cellulose or a derivative thereof or agarose, a resin, a fatty alcohol, fatty acid and esters thereof, a mineral such as silica or a silicate, kaolin, bentonite, diatomaceous earth, vermiculite, pumice or mineral wool, and a vegetable material such as wheat bran or seeds, e.g. poppy or sesame seeds.

The composition may also be formulated as a coated tablet or capsule comprising the fungal material, the coating being adapted to dissolve or disintegrate in the intestines to release the fungal material. The composition may, however, advantageously comprise coated granules containing the fungal material as these are usually easier to administer since they can be admixed with the feed dispensed to the animals.

The coating employed to protect the fungal material is preferably an enteric coating which is usually a coating soluble at the pH prevailing in the large intestine or in the faeces, i.e. typically a pH of 7 or more. Examples of enteric coating materials are selected from shellac, cellulose acetate esters such as cellulose acetate phthalate, hydroxypropyl methyl cellulose esters such as hydroxypropyl methyl cellulose phthalate, polyvinyl acetate esters such as polyvinyl acetate phthalate, and polymers of methacrylic acid and (meth)-acrylic acid esters.

Alternatively, the coating may be one which is enzymatically degradable in the intestines, especially the large intestine, or in the faeces, or a water-permeable coating which may be selected from ethyl cellulose, cellulose acetate, cellulose propionate, cellulose butyrate, cellulose valerate, polyvinyl acetate, polyvinyl formal, polyvinyl butyral, polymethyl methacrylate, polycarbonate, polystyrene, polyester and polybutadiene. The water-permeable coating is, however, preferably a microporous membrane, for instance selected from polyvinylchloride, and microporous polycarbonates, polyamides, acrylic copolymers and polyurethanes which permit a gradual diffusion of the fungal material, and if a water-permeable coating is used it is preferred to combine it with an enteric coating so that diffusion of the fungal material is substantially prevented under gastric conditions.

When the composition is to be administered to ruminants, it may be advantageous to provide a futher layer of coating as the coated granules containing the fungal material. This should be a coating which prevents the inner coating layer from dissolving in the rumen (this may for instance occur when the inner coating layer is an enteric coating which is dissolved at a pH of about 7 which is the pH of the ruminal contents). Such an outer coating layer may comprise a coating

material which is soluble at an acid pH such as a pH of about 1.5-2.5.

It may also be possible to provide a further coating layer on the composition of the invention, apart from the coating layer(s) providing the desired release characteristics as described above. Such a coating layer serves to improve the handling properties, storage stability, etc. of the composition. For the latter purpose, hydroxypropyl methyl cellulose may be used.

When the animals to which the composition is to be administered are ruminants, a preferred form of the composition is a bolus as it permits administration of the fungal material at long intervals while ensuring a continuous release from the bolus of an amount of fungal material sufficient to obtain a satisfactory nematode control. The bolus may suitably be of the matrix type and may either be one which slowly dissolves in the rumen or which is gradually eroded in the rumen. Suitable bolus materials may be selected from a natural or synthetic wax, a plastic, a polymer (e.g. carboxymethyl cellulose or polyvinylpyrrolidone), a polysaccharide, such as an alginate, a resin, a fatty alkohol, fatty acid and esters thereof and a glass. The fungal material may conveniently be embedded in the bolus in the form of coated granules in order to protect the fungal material from ruminal fluids which are indicated (cf. Example 10 below) to be particularly detrimental to the viability of the fungal material.

The bolus may be prevented from leaving the rumen by its shape in accordance with well-known practice or by including in the bolus matrix an agent which imparts an increased specific gravity to the composition, e.g. barium sulphate, titanium oxide, a zinc oxide or an iron salt.

The amount of active fungal material incorporated in the bolus composition may vary within wide limits, but is suitably adapted to the amount of fungal material which it is desired to provide in the faeces during the critical period as explained above. Thus, an adequate amount of fungal material in a bolus may be in the range of 1-15 g, in particular 2-10 g, per bolus, *inter alia* dependent on the

rate at which the bolus disintegrates in the rumen which, in turn determines the interval at which a new bolus should be administered.

Where herbivores are concerned (also other than ruminants), the composition of the invention may also be in the form of a feed block containing embedded coated granules comprising the fungal material. This form of the composition may, however, be less preferred since the amount of fungal material ingested by the animals by block feeding will not be uniform, depending on a variety of factors such as the availability of grazing or other feed or, most importantly, the variations in the individual uptake of the fungus from the feed block (some animals being less willing to avail themselves of the feed block than others). If a feed block is to be employed, the block material may for instance comprise molasses as this may improve the palatability of the block and thereby contribute to more frequent use thereof by the animals.

In accordance with the principle of the invention it may be an advantage if the composition further comprises a chemical antiparasitic agent, such as an anthelmintic as discussed above, or another active agent such as a growth promoting agent, hormone, vitamin, micro- or macronutrient or amino acid. Any such further active agents may be incorporated in the composition in accordance with accepted practice in the field of veterinary pharmacology.

It has surprisingly been found that the fungi from which the fungal material employed in the method and composition of the invention is derived may be grown under submerged conditions.

Accordingly, the present invention further relates to a process for producing a fungal material of a nematode-destroying fungus, which process comprises

a) inoculating a suitable liquid medium with mycelium or spores of the fungus,
b) growing the fungus in submerged culture under aeration and agitation to produce a fungal material, and
c) harvesting the fungal material.

Thus, it has become possible to produce the fungal material by slightly modifying standard fermentation techniques using submerged cultivation described in, for instance, *Bergey's Manual*, and large-scale production of nematode-destroying fungi for controlling not only animal parasitic nematodes, but also plant parasitic nematodes has therefore become more economic than when employing the methods of growing the fungus suggested in the known art.

The process may be conducted under the ususal conditions for anaerobic cultivation, such as in the presence of a suitable carbon source, e.g. selected from a sugar, e.g. sucrose, lactose, glucose, maltose, xylose, fructose, galactose, a starch, sodium lactate, malt extract, glucose syrup and lactose permeate, and a suitable nitrogen source, e.g. selected from asparagine, yeast extract, $NaNO_3$, Bacto peptone, corn steep liquor and casamino acids.

The fungal material is suitably harvested by filtration or centrifugation according to standard procedures, and the harvested mycelium may then be dried according to usual methods such as by means of dry air, e.g. in a fluid bed, in vacuum, by freeze-drying or by dessication with a suitable dessicant such as anhydrous magnesium sulphate, silica gel, etc.

The fungal material produced by the process of the invention may be derived from any of the fungal genera or species indicated above.

In one particular embodiment of this method, the medium comprises a vegetable, organic or inorganic solid support to which the fungus is able to adhere and on and/or in which it will grow. The solid support may suitably comprise a spongy or porous material such as seeds, e.g. poppy or sesame seeds, or an organic or inorganic polymer such as porous polyacrylic or glass beads.

It is, of course, also possible to produce the fungal material by growing the fungus under aerobic conditions on a solid medium, the fungal material being either harvested from the medium or, if the medium is edible in itself and/or if the the concentration of fungal material is sufficiently high, the medium containing the fungal material may be used as such.

When using a solid medium for fermentation of the fungus, the medium may suitably be inoculated with propargyles (the smallest segment capable of sporulation) of the fungal mycelium. If it is desired to produce a fungal material containing a large quantity of spores, the mycelium is subjected to a gradual drying-out process in air after propagation of the fungus as this has been found to increase sporulation.

It is generally preferred to harvest the fungal material after propagation since this ensures a higher concentration thereof and makes it possible to formulate it into a composition. Furthermore, it may be difficult to obtain a sufficient storage stability of the fungal material if the depleted medium is not removed and to avoid contamination of the resulting product by other organisms using the residual medium as substrate. Harvesting of the fungal material may be performed by scraping, washing, filtration, centrifugation or a combination of two or more of these procedures, optionally followed by drying.

The solid medium on which the fungus is grown may be any medium which has been found to favour the growth and/or sporulation of this type of organism. A suitable medium according to this criterion is one which contains a cereal component such as corn.

The present invention further relates to a method of reducing the number of infectious nematodes in the environment of animals so as to reduce the transmission of nematode infections to animals inhabiting said environment, the method comprising administering to an animal a composition comprising a fungal material produced by submerged cultivation as described above in order to provide an adequate nematode-controlling amount of the fungal material in the animal faeces. Although the fungal material is preferably in protected form as indicated above, it may also be formulated into a composition where the fungal material is not protected. This approach may be applicable for fungal species or strains which are found to be sufficiently resistant to gastrointestinal conditions so that protection which will inevitably increase the cost of the composition may be omitted. In such cases, the fungal material may be formulated as granules to be

admixed with the feed or spread over a pasture or an indoors enclosure for animals, or embedded as such in a bolus or feed block.

The following examples serve to illustrate the invention in further detail, but are not intended to limit the invention in any way.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1a-1d show the number of loops per mm^2 and trapping efficiency of *A. oligospora* exposed to first-, second- and third-stage *C. oncophora* larvae and to *R. wohlgemuthi* (juveniles and adults). The average number of free individuals in fungus-containing dishes is expressed as a percentage of that in fungus-free control dishes. Black dots denote freely migrating nematodes in per cent of controls, and white dots denote the number of hyphal loops per mm^2.

Figs. 2a-2d show the number of loops per mm^2 and trapping efficiency of *A. oligospora* exposed to first-, second- and third-stage *C. oncophora* larvae and to *P. redivivus* (juveniles and adults). The average number of free individuals in fungus-containing dishes is expressed as a percentage of that in fungus-free control dishes. It should be noted that the figure on the scale for *P. redivivus*-induced loops are higher than those of the other scales. Black dots denote freely migrating nematodes in per cent of controls, and white dots denote the number of hyphal loops per mm^2.

Fig. 3 shows the concentration of infective *C. oncophora* larvae in cowpats and surrounding grass at different times. • denotes addition of *A. oligospora* to the faeces and ▲ denotes no addition of *A. oligospora* to the faeces.

Figs. 4 and 5 show the concentration of infective *C. oncophora* larvae (L_3) per gram of faeces either containing (+) or not containing (°) *A. oligospora*.

EXAMPLE 1

Growth of Arthrobotrys oligospora on cornmeal agar and harvesting of spores

Cornmeal agar plates containing 0.5 % of dextrose were inoculated with a 0.5 ml spore suspension of *A. oligospora* Fresenius (ATCC 24927) (used throughout the Examples). 22 days later, 1 x 1 cm segments from the plates (containing mycelium and spores) were used to inoculate plates (9 cm in diameter) containing 2.7 % cornmeal agar (CMA; 77 % of cornmeal) media of different compositions:

A: 1,7 g of CMA, 13.5 g of Bacto agar, 2 g of K_2HPO_4, distilled water ad 1 l
B: 8.5 g of CMA, 11.8 g of Bacto agar, 2 g of K_2HPO_4, distilled water ad 1 l
C: 17.0 g of CMA, 10.0 g of Bacto agar, 2 g of K_2HPO_4, distilled water ad 1 l
D: 25.0 g of CMA, 8.1 g of Bacto agar, 2 g of K_2HPO_4, distilled water ad 1 l

The plates were incubated at 26°C in the dark for 15 days and at 19-23°C and 12 hours of light/12 hours of darkness a day for 14 days.

In a separate experiment, different concentrations of glucose (0 g/l, 2 g/l, 5 g/l and 10 g/l) were added to the CMA plates which were inoculated and incubated as described above.

It appeared from the results that the growth intensity (biomass) varies greatly according to the CMA concentration employed, as follows:

A: weak growth
B: slightly more abundant growth
C: abundant growth
D: very abundant growth

No differences in growth intensity were observed at different glucose concentrations.

It further appeared that sporulation increased with increasing CMA concentrations, whereas sporulation decreased as a result of increasing glucose concentrations. It was therefore concluded that no glucose should be added to the growth medium for *A. oligospora*.

It was further shown that sporulation was improved when the fungus was grown under conditions of 12 hours of light/12 hours of darkness a day than constantly in the dark or in the light.

EXAMPLE 2

Harvesting of Arthrobotrys oligospora spores from CMA plates

Plates containing a CMA medium with the following composition: 17 g of CMA, 5 g of Oxoid agar, 2 g of K_2HPO_4, 1 l of water, were inoculated and incubated as described above (26°C, 12 h light/12 h darkness/day) until the plates were completely covered with mycelia and spores.

The spores were harvested by 1) washing the plates with water, paraffin oil, 96% ethanol or water containing 0.1% of Tween® 80, or 2) scraping off the spores by means of spatula without adding any liquid.

Procedure 1) was performed by adding 10 ml of washing liquid to the plates, loosening the spores by means of a spatula, filtering the spores on a Büchner funnel through a filter with a pore size of 8 µm or centrifugating the spores at 6000 rpm., drying the spores in a dessicator or by means of dry air at room temperature and storing the spores in a refrigerator or at room temperature.

The spores obtained by procedure 2) were dried in a desiccator or by means of dry air at room temperature and stored in a refrigerator or at room temperature.

The spores obtained by procedure 1) were subjected to a viability test by cutting the spore-containing filters into smaller pieces, placing the pieces in tubes and adding 2 ml of water. The tubes were vortexed, and a few drops were pipetted off and inoculated onto a

CMA-9 plate. It was practically impossible to remove the spores from the filters resulting from the paraffin wash, so that no viability test could be carried out in this case. A sufficient amount of water was added to the tubes in which spores had been centrifugated to suspend the spores, and a few drops of the suspension were used to inoculate CMA-9 plates.

The spores obtained by procedure 2) were tested for viability by suspending a loopful of the spores in water and inoculating them onto CMA-9 agar plates.

24 hours after inoculation, the plates were examined for germinated and non-germinated spores and the viability of the spores was calculated.

TABLE 1

Procedure 1 (filtration)	Drying	Storage	% viability after 1 week	% viability after 3.5 months
H_2O	Dessicator	Room temp.	82	0
H_2O	Room temp. (dry air)	Room temp.	3	0
H_2O	Dessicator	Refrigerator	96	88
H_2O	Room temp.	Refrigerator	2	5
H_2O + Tween® 80	Dessicator	Room temp.	73	0
H_2O + Tween® 80	Room temp.	Room temp.	96	0
H_2O + Tween® 80	Dessicator	Refrigerator	96	14
H_2O + Tween® 80	Room temp.	Refrigerator	95	29
96% C_2H_5OH	Dessicator	Room temp.	0	0
96% C_2H_5OH	Room temp.	Room temp.	0	0
96% C_2H_5OH	Dessicator	Refrigerator	0	0
96% C_2H_5OH	Room temp.	Refrigerator	2	0

Procedure 1 (centrifugation)	Drying	Storage	% viability after 1 week	% viability after 3.5 months
H_2O	Dessicator	Refrigerator	81	93
H_2O	Dessicator	Room temp.	83	5
H_2O	Room temp.	Room temp.	30	3

Procedure 2	Drying	Storage	% viability after 1 week	% viability after 3.5 months
Scraping off the spores without liquid	Dessicator	Room temp.	98	3
	Room temp.	Refrigerator	99	97

It appears from Table 1 that when using procedure 1), the best results are obtained by using water as the washing liquid, drying the spores in a dessicator and storing the spores in a refrigerator. However, procedure 2) is currently preferred as it comprises fewer steps and leads to a viability of nearly 100% after one week, which has only decreased by 2% after 3.5 months.

EXAMPLE 3

Growth of Arthrobotrys oligospora on crushed corn

Crushed corn was obtained by rolling, chopping or blending and sieved to a particle size fraction of about 1.25 mm. The crushed corn of a substantially uniform particle size was autoclaved for 30 minutes at 121°C and left in the autoclave overnight. 20 g of this medium was placed in petri dishes with a diameter of 9 cm. To the medium was added 50-150% of water (calculated on the raw weight of the corn).

The dishes were inoculated with segments of *A. oligospora* mycelium, substantially as described in Example 1. The dishes were then incubated in the dark at 26°C.

Growth of the fungus was determined as radial growth, i.e. the area of the petri dish covered by the fungus, and the growth intensity was graded as follows:

1: weak growth
2: medium growth
3: abundant growth

Furthermore, the growth intensity of the fungus was determined by the degree to which the mycelium had grown through the substrate and was visible at the bottom of the petri dish. This was evaluated as follows:

+: some mycelium was visible
++: more mycelium was visible
+++: much mycelium was visible

The latter effect (+++) was observed after 12 days of incubation.

The results are shown in Table 2 below.

TABLE 2

	% of water	Day 5	8	12
Rolled corn	50	1	1,+	1,+
	70	1,5,+	2,+	2,+
	90	3,+	3,+	3,+++
	110	3,+	3,+	3,++
	130	3,+	3,+	3,+++
	150	3	3,+	3,+
Chopped corn	50	1	1	1
	70	2,+	2,+	2,+
	90	3,+	2,+	2,++
	110	3,+	3,+	3,+++
	130	3,+	3,+	2,5,++
	150	3,+	3,+	3,+
Blended corn	50	1	1,+	1,+
	70	2	2,+	2,5,+++
	90	2,5	3,+	3,+++
	110	3,+	3,+	3,++
	130	3,+	3,+	3,++
	150	3	3,+	3,++

The data shown in Table 2 are the mean of two experiments.

It appears from Table 2 that the best growth of *A. oligospora* is obtained with a content of water in the medium of more than 90%. Similarly, the data for growth of the mycelium through the substrate show that the optimum content of water for obtaining abundant growth is in the range of 90-110%. Furthermore, good sporulation was observed at this recommended content of water.

EXAMPLE 4

Growth of Arthrobotrys oligospora in submerged media

CMA plates were inoculated with *A. oligospora* and incubated as described in Example 1. After 20 days, the plates were washed with 10 ml of sterile water, and the water containing the fungal material was used to inoculate shaking flasks (one plate per flask) containing 100 ml of nutrient medium of the following composition:

Czapek Dox medium: 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4$, 1.5 g of $CaCl_2$; 1 l of distilled water, 1 ml of 1 g of $ZnSO_4$/100ml of H_2O, 1 ml of 0.5 g of $CuSO_4$/100 ml of H_2O;

7.56 g of corn steep liquor (shown to be an advantageous nitrogen source in a previous experiment - not reported here - in which several nitrogen sources had been tested); and

35 g of malt extract (shown to be an advantageous carbon source in a previous experiment - not reported here - in which several carbon sources had been tested).

The flasks were incubated at 26°C on a shaking apparatus in the dark for one week. The contents of two flasks were used to inoculate a 10 l fermenter (Braun) containing 7 liters of the nutrient medium defined above, corresponding to about 8-10 g of wet weight of *A. oligospora*.

The fermenter was incubated for 3 days at 26 ± 1°C, maintaining a cycle of 12 hours of light/12 hours of darkness/day. The initial pH in the fermenter was 5.8 which was maintained at this level by titrating with a 5N NH_3 solution, using 100 ml in 3 days. The fermenter was aerated by means of an oxygen flow of 5.5 l/minute and rotated at 750 rpm. To prevent foaming, 5% of an antifoaming agent was added, using 100 ml in 3 days. After 3 days, 6N HCl was added to a pH of 7.0.

The mycelium formed after 3 days was filtered off by means of a Büchner funnel.

The yield of mycelium (it should be noted that no sporulation was observed when the fungus was grown in submerged media) was about 700 g wet weight (i.e. about 10%). The fungus showed a filamentous growth which is particularly well suited for further processing; the filamentous growth is believed to be caused by the rotation at 750 rpm. All of the sugar in the malt extract had been consumed in 3 days, and it must be assumed that a more concentrated nutrient medium will lead to increased yields of mycelium.

EXAMPLE 5

The temperature optima of different predatory fungi

Plates containing a CMA medium of the following composition : 17 g of CMA, 5 g of oxoid agar, 2 g of K_2HPO_4, 1 l of water, were inoculated with 1 x 1 cm segments of agar plates incubated as described in Example 1 with the following organisms:

Arthrobotrys oligospora
A. oligospora Swart
A. oligospora Drechsler
A. conoides
A. superba
A. arthrobotryoides
A. musiformis
A. tortor
Dactylaria candida

The plates were incubated at 12 hours of light/12 hours of darkness at 15, 20, 30, 37 and 40°C.

The temperature optima of the different organisms were determined in terms of the maximum diameter of the growth zone. *A. arthrobotryoides* had a temperature optimum at 20°C, *A. tortor* had a temperature optimum at 30°C, and all the other organisms tested had a temperature optimum at 25°C.

EXAMPLE 6

Predacious activity of Arthrobotrys oligospora on preparasitic larvae of Cooperia oncophora and on soil nematodes

The strain of *A. oligospora* Fres. (ATCC 24927) was grown and maintained on corn meal agar (CMA) adjusted to pH 7 as described by Lysek and Nordbring-Hertz (1981) (42). This medium allowed good mycelial growth and formation of conidia but not trap development. Test Petri dishes, 3.2 cm in diameter, were each filled with approximately 4 ml of CMA and inoculated with 3-5 weeks old fungal cultures. Using a metal cork borer (5 mm in diameter), circular agar plugs were cut from the fungal lawn and placed with mycelium down in the centre of the CMA Petri dish.

Soil nematodes: *Panagrellus redivivus* was cultured in flasks containing a soy peptone-liver extract medium (Nordbring-Hertz, 1972 (43)). *Rhabditis wohlgemuthi* was cultivated on serum agar plates (Monrad, pers. comm.). These nematodes were harvested from approximately one week old cultures, using the Baermann funnel technique, and washed several times by alternate centrifugations and resuspensions in sterile water. The resulting suspensions contained both adult and juvenile nematodes.

Parasitic nematodes: Eggs of *Cooperia oncophora* were harvested from the faeces of a calf carrying an experimental monospecific infection of the nematode. Larvae were allowed to develop in the faeces by a cultivation procedure of Henriksen and Korsholm (1983) (44) and were isolated by a modified Baermann technique. By starting cultures at different intervals, it was possible to have batches of L_1, L_2 and L_3 larvae available simultaneously. The three external developmental stages of the larvae are L_1 and L_2 in which the larvae are pre-infective and feeding on bacteria, and L_3 in which the larvae are encased in the cast cuticle of the second moult. In each case, the stage of development was checked by microscopic examination. Prior to use in the experiments, the larvae were washed by serial centrifugations and resuspensions in sterile water.

To compare the ability of the nematodes to induce capture organs (hyphal loops) and to become trapped in such organs, suspensions thereof were added to 4-day old cultures of *A. oligospora*. A drop of each nematode suspension adjusted to 100-150 nematodes per drop was added to each of three test and three control dishes. Counts of free-living nematodes included both adults and juveniles. To check whether any nematode-free substance of the inoculum would induce traps, fungus CMA dishes were exposed to one drop of the supernatant of each of the final nematode suspensions.

Starting with 3-hour intervals and ending with 6-hour intervals, for a 27-hour period, test dishes were examined at 100 x using a binocular microscope. Trap formation began as a stout branch erecting from a vegetative hypha. Subsequently it grew and curled back so its tip anastomosed with the parent hypha or with an adjacent trap already formed. Only traps which formed completely closed loops, either in isolated position or more commonly as part of complex, three-dimensional networks, were counted. Under the microscope, five randomly selected fields were counted in each dish giving a total of 15 fields. The average number of traps per mm^2 was calculated.

At each time interval, nematodes were also counted (x 20). Only the normal, freely moving individuals were enumerated. In order to account for "natural" deaths among the nematodes, the average numbers of such free individuals recorded in the test dishes were expressed as a percentage of those in fungus-free control Petri dishes. In addition, on several occasions the contact between individual worms and traps was studied in closer detail with higher magnification (100 x).

Two series of experiments were conducted: Series A included *R. wohlgemuthi* and the three developmental stages of *C. oncophora*. Series B differed in that *P. redivivus* replaced *R. wohlgemuthi*. In addition, at the end of series B, the third stage (L_3) *C. oncophora* larvae were added to the dishes where traps were already present and induced by the other nematodes.

Fig. 1 (Series A) and Fig. 2 (Series B) show that the rates of trap development were virtually independent of the type of nematode added.

After 3 hours, traps were induced in some of the nematode dishes, but at 6 hours traps were formed in all nematode-fungus combinations. Over subsequent hours, all dishes exhibited an almost parallel increase in number of traps. The dishes receiving nematode-free supernatants had no traps.

Also, Figs. 1 and 2 show that the decline in the numbers of the free migratory nematodes started 3-6 hours after they were added to the dishes. This coincided with the initiation of trap formation. After 9 hours, the majority of the nematodes were trapped and at 15 hours, there was an almost complete absence of freely migrating individuals. Checks using higher magnification (100 x) revealed that the majority of immobile nematodes were trapped. However, *R. wohlgemuthi* (Series A) seemed to present an exception in that a few migratory juveniles were observed at the end of the experiment.

On close examination, all treatments showed nematodes migrating over the entire agar surfaces of the dishes. On the fungus dishes, they migrated in close physical contact with the hyphal networks and occasionally caused slight movements of the hyphal system. After the traps had developed, it was noticed that casual nematode contacts did not necessarily result in immediate capture. Sometimes nematodes were seen to move into the loops and then escape by suddenly retracting, curling up and circumventing the trap. All nematodes exhibited this interesting behaviour but inevitably most were trapped.

However, once the preys were ensnared, their subsequent fate was sealed. Free-living nematodes and L_1 and L_2 stages of *C. oncophora* wriggled for up to a few hours after which they became paralyzed, and fungal hyphae could be seen inside their bodies. The L_3 stage of *C. oncophora*, on the other hand, struggled vigorously for a much longer period of time, and a few of them even succeeded in breaking the hyphal nets. Single individuals were seen freely moving on the substrate with fungal loops aroung their bodies with attached hyphal branches. Before long, these nematodes were recaptured in other traps. The L_3 of *C. oncophora* continued to wriggle in their traps, some for more than 20 hours after their capture.

In Series B when the original nematodes were all caught, a suspension of L_3 *C. oncophora* was added to one of each type of test dishes and to one control dish. Fig. 2 shows that all test dishes possessed a rapid and high trapping efficiency in that all nematodes were caught within approximately one hour. In the previously *P. redivivus* exposed dish, traps were particularly numerous and the capture of L_3 was instantaneous. On closer inspection, it was again noticed that larvae struggled violently, but after 24 hours roughly only 15% remained active.

EXAMPLE 7

Field experiments on the ability of Arthrobotrys oligospora to reduce the number of larvae of Cooperia species in cow pats and surrounding grass

Arthrobotrys oligospora Fres. (strain ATCC 24927) was cultured on 20 g of chopped maize grains and 18 ml of H_2O autoclaved in Petri dishes (diameter 8.5 cm). Cultures were maintained at 23°C and 95-100% humidity for 2 months at which time the fungus had developed a dense mycelium with conidia on the surface of the medium.

Faeces containing parasite eggs were obtained from a calf experimentally infected with a monoculture of *Cooperia* spp. Infective *Cooperia* spp. larvae were isolated from faeces by a modified Baermann technique (Jørgensen & Madsen, 1982 (45)). Infective *Cooperia* spp. larvae were isolated from grass by the agar technique described by Jørgensen (1975) (46) and Mwegoha & Jørgensen (1977) (47).

Recordings of the weekly maximum and minimum mean temperatures 2 m above the ground level and the total weekly precipitation were obtained at a station 300 m from the experimental field.

Experiment 1:

Approximately 10 g of mycelium with conidia were carefully isolated from the Petri dishes containing *Arthrobotrys oligospora*. The fungal material was thoroughly mixed with one of two 1 kg faecal portions containing an even distribution of 300 *Cooperia* spp. eggs per gram.

Both faecal portions were, in the form of cow pats (diameter 18 cm), placed on a parasite-free pasture in May. Samples of approximately 2 g of faeces were taken from the edge of the cow pats at intervals. Grass was sampled within a distance of 0-20 cm from the edge of the pats. The first half circle was harvested 29 days after the start of the experiment. The last half circle was taken after 57 days. Times of faeces and grass samplings are shown in Table 3. The concentration of infective *Cooperia* spp. larvae in faeces and grass was subsequently determined.

Experiment 2:

In this experiment, the inoculation material comprised both *Arthrobotrys oligospora* and its growth medium, in that the whole contents of Petri dishes were milled in a mincing machine. The milled material was subsequently divided into 10 portions of 150 g (dry weight 56 g), which were added to each of 10 faecal portions of 1 kg. Another 10 faecal portions of 1 kg were kept as fungus-free controls. All the faecal portions, which contained a uniform distribution of 560 *Cooperia* spp. eggs per gram, were placed as cow pats (diameter 18 cm) on a parasite-free pasture in June.

The minimum distance between the two groups was 2.5 m and the distance between single cow pats in each group was 1.7 m.

At intervals samples of approximately 1 g of faeces were taken from the edge of the cow pats and at the same occasions, grass was harvested at different locations around the pats. Grass samples were cut in swaths of 7.5 cm in width at a distance of 20 cm from the edge of the cow pats. Samples of faeces and grass from each group of cow pats were pooled, and the concentration of infective *Cooperia* spp. larvae ir the total samples of faeces and grass was determined. The time of samplings is shown in Fig. 3.

The inoculation in experiment 2 was rather large (150 g per 1000 g of faeces) and this itself could perhaps damage the composition of the habitat, e.g. its water content. Therefore, on day 21 and 36, 10 g of faeces from the two groups of cow pats were obtained and dried at

105°C for 48 hours, and the percentage water content was measured as: (Water loss (g)/weight of dry faeces (g)) x 100%.

The results of experiment 1 are presented in Table 3. During the first 25 days of the experiment, there was a marked reduction in the number of infective *Cooperia* spp. larvae in the cow pats mixed with mycelium and conidia of *Arthrobotrys oligospora* and in consonance with this, the transmission of infective larvae to the surrounding grass was significantly reduced as compared with the transmission from control pats. On day 29, the relative reduction in grass contamination amounted to 96%.

TABLE 3

The effect of admixture of the fungus *Arthrobotrys oligospora* on the concentration on infective *Cooperia* spp. larvae in the cow pats (faeces) and in the surrounding grass at different time intervals after the start of the experiment

		Concentration of infective *Cooperia* larvae (L_3)			
		FAECES (L_3/g)		GRASS (L_3/kg(dry))	
Date	Day No.	+fungus	-fungus	+fungus	-fungus
14-5-1984	0	0	0	0	0
23-5-1984	9	0	0	-	-
1-6-1984	18	0	155	-	-
8-6-1984	25	1	40	-	-
12-6-1984	29	-	-	630	14480
10-7-1984	57	-	-	28	2434
30-7-1984	77	0	0	-	-

-: Not determined.

Results from the more comprehensive experiment 2 are shown in Fig. 3. At day 14, infective *Cooperia* spp. larvae were recorded in the faeces. Over the following weeks, larvae were gradually released to the surrounding grass reaching a maximum concentration on day 29. Apparently, larvae were spread during the period from 14 to 29 days after the start of the experiment, probably facilitated by the rainy weather in this period.

The presence of *Arthrobotrys oligospora* in the cow pats resulted in a marked reduction in the number of infective *Cooperia* spp. larvae in both the cow pats and surrounding grass as compared with the controls (Fig. 3). On day 29, *A. oligospora* was responsible for an 86% reduction in the number of infective larvae in the grass.

In experiment 2, the water content in cow pats with and without the milled fungal material was 268% and 289% on day 21 and 376% and 355% on day 36. It is reasonable to conclude that the water content of the two types of cow pats was sufficiently similar not to account for differences in the composition of the faecal habitats and finally for differences in numbers of *Cooperia* spp. larvae developing in the two types of pats.

In both experiments, the nematode-trapping fungus could be demonstrated in the inoculated cow pats using the sprinkling technique on corn meal agar (Fowler, 1970 (48)).

EXAMPLE 8

Field experiments on the ability of Arthrobotrys oligospora to reduce the number of larvae of Ostertagia ostertagi in cow pats and surrounding grass as well as to reduce the extent of parasite infection in calves

Faeces containing eggs of the parasite *Ostertagia ostertagi* was obtained from donor calves with experimental monoinfections. The collected faeces was immediately cooled to 5°C to prevent the eggs from developing. The day before cow pats were placed in the test pasture, portions of about 25 kg of faeces were mixed in a cement mixer at

room temperature for 1-1.5 hour in order to secure a uniform distribution of *O. ostertagi* eggs. This material was subsequently divided into 24 portions of 1 kg of faeces which were moulded into cow pats with a diameter of 16 cm and a height at the centre of 3 cm. Prior to deposition in the pasture, 12 of these experimental cow pats were inoculated with 2 million *A. oligospora* conidia per 1 kg of faeces (that is 2000 conidia/g) in a 10 ml aqueous suspension. 10 ml of fungus-free water had been added to the remaining cow pats which were used as controls.

The fungus-inoculated and control cow pats were placed at intervals of 2.6 m in separate plots of pasture. The procedure described above was repeated three times so that the experimental cow pats were placed in the plots for four consecutive months at intervals of about four weeks. The cow pats were protected against dung-seeking birds by means of hemispherical nets with a diameter of about 50 cm and a mesh diameter of 6 cm.

At intervals of 14 days, samples of a few grams of faeces were collected from the edge of the cow pats, and samples of grass were cut from the edge of the cow pats to a distance of 20 cm and in swaths of 7 cm. The grass was cut as close to the ground as possible.

The number of infective *O. ostertagi* larvae per gram of faeces (LPG) was determined by a modified Baermann technique (Jørgensen and Madsen, 1982 (45)). Infective larvae in grass were isolated by the agar gel technique developed by Jørgensen (1975) (46) and Mwegoha and Jørgensen (1977) (47).

In the final stage of the experiment, parasite-free "tracer" calves were turned to grass for two weeks in the two plots, with and without fungus-inoculated cow pats, approximately two weeks after the last portion of cow pats had been placed in the pastures. The calves were then stalled for three weeks until they were slaughtered. The concentration of serum pepsinogen in the blood from which the extent of impairment of the gastrointestinal mucosa caused by the parasites may be determined, was measured. The tracer calves were slaughtered and the number of parasitic nematodes in the abomasum were recorded.

Recordings of the weekly maximum and minimum temperatures two metres above ground level and the total weekly precipitation were obtained at a weather station about two kilometers southeast of the experimental plots.

Results

Cow pat samples

The results from the cow pat samples are shown in Figs. 4 and 5. The rather low EPG (eggs per gram of faeces) in cow pats placed in the pastures in May and June together with a relatively dry period in both these months resulted in low concentrations of L_3-larvae in these pats during the entire experimental period. The higher EPG in cow pats placed in July and frequent showers in July and August resulted in a greatly increased development of larvae in these cow pats (Fig. 5).

The results of the samples from the cow pats showed that there was a significantly smaller number of infective *O. ostertagi* larvae in cow pats admixed with conidia of *A. oligospora* than in the control cow pats. This pattern was particularly pronounced in cow pats which had been placed in the pastures in July, where a reduction in the number of L_3-larvae of 63% was observed in cow pats admixed with the fungus when measured four weeks after the cow pats had been placed in the pasture, cf. Fig. 5.

Grass samples

The number of infective larvae of *O. ostertagi* around the cow pats was initially very low due to the dry weather in the early season. After about six weeks when the weather became more rainy, larvae were gradually released to the surrounding grass so that the maximum of L_3-larvae measured in the grass around the control cow pats reached the highest level at approximately the same time, namely 12, 8 and 4 weeks, respectively, after they had been placed. At this point, the largest reduction in the number of L_3-larvae in the grass around cow pats admixed with conidia of *A. oligospora* compared with the controls

of 48%, 89% and 46%, respectively, for cow pats placed in May, June and July could be observed.

Tracer calves

The results from the tracer calf experiment are summarized in Tables 4 and 5.

TABLE 4

Pasture +/- fungus	Animal No.	Serum pepsinogen (tyrosin/liter) Week 0	1	2	3	4	5
+	260	0.4	0.9	0.9	1.0	0.6	0.7
	262*	0.3	0.8	0.8	1.7	2.4	1.6
	741	0.4	0.6	0.7	1.0	1.0	0.7
	259	0.2	0.4	0.6	0.5	0.5	0.4
	X	0.3	0.7	0.8	1.1	1.1	0.9
-	263	0.3	0.7	0.7	0.9	0.7	0.7
	258	0.4	0.9	0.9	0.9	1.0	1.2
	256	0.6	0.7	0.9	0.8	0.8	1.0
	261	0.5	1.0	1.0	1.3	1.4	1.3
	X	0.5	0.8	0.9	1.0	1.0	1.1

95% of healthy animals will show values of between 0.3 and 0.7.

TABLE 5

Pasture +/- fungus	Animal No.	Number of *O. ostertagi* in the abomasum at slaughtering (week 5)
+	260	2680
	262	26000*
	741	8170
	259	3040
	X	9973
-	263	7980
	258	11350
	256	11280
	261	13040
	X	10913

*) aberrant grazing behaviour.

It appears from Tables 4 and 5 that calf No. 262 (marked with an asterisk) deviated from the other calves in the group grazing in the fungus-treated plot in that it showed surprisingly high serum pepsinogen values (Table 4) as well as the largest number of nematodes in the abomasum (Table 5). The unexpected high values from calf No. 262 were responsible for the fact that no statistically significant difference between the two groups of calves could be demonstrated during the test period, and the fact that no statistically significant difference in the number of adult *O. ostertagi* individuals in the abomasum (worm counts) could be observed.

Based on these observations, it is suggested that calf No. 262 had an abnormal grazing behaviour in that it seemed to ingest the highly contaminated grass close to the cow pats. This grass is normally avoided by calves as long as there is no general grass depletion in the pasture. If, therefore, the data from this calf presented in

Tables 4 and 5 are deleted, a significant beneficial effect is obtained by adding viable *A. oligospora* in cow pats both with respect to serum pepsinogen levels (0.6 in week 5) and worm counts (4630).

Furthermore, it should be noted that the tracer calves had been turned to grass at a time when the infective level of *O. ostertagi* in fungus-treated and fungus-free plots approached comparable levels. This, too, might have contributed to the very small difference between the two groups of tracer calves. If the tracer calves had been turned to grass two weeks earlier, the calves grazing in the fungus-treated plot would very likely have ingested a far smaller number of nematode larvae than the calves grazing in the fungus-free plot.

EXAMPLE 9

Interactions between Arthrobotrys oligospora and third-stage larvae of a number of animal parasitic nematodes

The ability of *A. oligospora* to develop trapping devices and to trap a variety of animal parasitic nematodes in the third infective larval stage was examined, and comparisons were made with indigenous soil nematodes.

The experiment included the following species of parasitic nematodes:

Cooperia oncophora, *Ostertagia ostertagi*, *Dictyocaulus viviparus* (infecting cattle), *Cooperia curticei*, *Haemonchus contortus* (infecting sheep), *Oesophagostomum dentatum*, *Oe. quadrispinulatum* (infecting pigs), *Cyathostoma* spp. (infecting horses) and *Nematospiroides dubius* (infecting mice). All species, except the cyathostomes which included a broad spectrum of species from a naturally infected horse, were obtained from animals with experimental mono-specific infections. Larvae were allowed to develop up to the third stage by standard cultivation procedures and were subsequently isolated by modified Baermann techniques. Before use, the larvae were washed by centrifugations and resuspensions in sterile water. Stage of development and motility were checked by microscopial examination.

The soil nematodes employed in this experiment were *Panagrellus redivivus* which was cultured axenically in flasks containing soy peptone-liver extract (Nordbring-Hertz, 1972 (43)) and *Rhabditis wohlgemuthi* which was cultured on bacteria-containing serum agar plates. Both species were subcultured once or twice a week. Before use in the experiment, they were harvested by baermannization and were subsequently washed by alternate centrifugations and resuspensions in sterile water. The final suspensions contained both adults and juveniles.

Arthrobotrys oligospora Fres. strain ATCC 24927 was used throughout the experiments. It was grown on corn meal agar (CMA) adjusted to pH 7 (Lysek and Nordbring-Hertz, 1981 (42)). To avoid contamination with bacteria and overgrowth of the dishes, benzylpenicilline and streptomycin sulphate were added to the agar in concentrations of 200 i.u. and 260 µg per ml, respectively. Approximately 4 ml of the agar were poured into each test petri dish with a diameter of 3.2 cm. The dishes were inoculated from stock culture dishes from which small blocks of agar were cut from the periphery of the actively growing colony and placed upside down on the new agar. The fungus dishes were exposed to daylight and kept at room temperature (20-23°C) and 98-100% of humidity. The mycelium usually reached the periphery of the dishes in 4-5 days. The dishes were supplied with a grid pattern on the bottom to facilitate nematode counting.

The study comprised two series of experiments, each including a variety of the parasitic nematode species and in addition one or both of the soil nematodes (Tables 6 and 7).

Trap development: For each of the tested species, a drop adjusted to contain approximately 500 nematodes was added to the centre of a 5-day old fungus dish. Drops of nematode-free supernatants of the respective batches were added to other fungus dishes to determine possible morphogenic potentials of substances not bound to the nematodes. A few fungus dishes in each series were left untreated. The dishes were examined at x 100 using a binocular microscope, the observations starting with 3-hour intervals and ending with 6-hour intervals over a 27-hour period. Finally, they were read at 48 hours. Only completely closed loops either in isolation or as part of a more

complex network were enumerated. Five randomly selected fields were examined per dish, and the average number of loops per mm^2 was enumerated.

Trapping efficiency: Pre-induction of trapping networks was made by adding approximately 500 *P. redivivus* to each of the 5-day old fungus dishes. After three days, numerous loops had formed, i.e. approximately 100-200 per mm^2 in series 1, and 350-500 in series 2. At this point, all nematodes were captured and consumed, having a more or less amorphous appearance. These dishes were used for assessing the trapping efficiency of *A. oligospora* against the various parasitic nematodes to be studied. Within 24 hours freshly added *P. redivivus* and other nematodes could easily be distinguished from the faint and partly dissolved *P. redivivus* that originally pre-induced traps on the dishes.

The experiment started with adding one drop, i.e. approximately 500 nematode individuals of a given species, to the centre of a dish containing traps pre-induced by *P. redivivus* as described above. In comparison, one drop was added to a fungus-free CMA dish. Nematodes were observed under a stereoscope (x 20) starting 30 minutes after adding the nematodes and then with the same intervals as described above for trap recordings. To account for deaths not related to fungal predacity, the number of normal, freely moving individuals on the test dishes was expressed as a percentage of those on the fungus-free control dishes. At intervals, the motility of the various nematodes and the behaviour of the captured individuals were studied in closer detail at higher magnification (x 100).

Results showing the ability of the different nematodes to induce trap formation in *A. oligospora* are summarized in Table 6. It appears that they were all able to induce traps within the period of observation, although at very different levels. No traps were formed on dishes treated with nematode-free supernatants or on the untreated dishes. Apart from *D. viviparus*, more than 80-90% of the larvae were motile at the time they were added to the dishes. Only 10-15% of the *D. viviparus* larvae were observed to move and most of the remaining larvae were coiled. In general, trap formation was much more pronounced

in series 2 than in series 1, both with respect to the lag period before the start of trap morphogenesis and to the final concentration of traps. Also, the pre-induced dishes of series 2 developed more traps than those of series 1 in response to the addition of *P. redivivus*. A possible explanation of this difference is that the mycelium in series 2, for some unknown reason, seemed to be more dense than in series 1. When comparisons were made within each of the two experimental series, it was found that the ability of the intestinal trichostrongyles of ruminants (*C. oncophora, C. curticei, H. contortus,* and *O. ostertagi*) and of the cyathostomes to induce traps was almost comparable to that of the soil nematodes and exceptionally higher than that of the porcine *Oesophagostomum* species and the murine *N. dubius*. The poorest trap inducer was *D. viviparus* which, even after a ten-fold increase of the inoculum (to raise the number of motile larvae to the level of motiles of the other nematode batches), only produced a few traps towards the end of the observation period. By observing the locomotive behaviour of the various nematodes on the dishes, it was evident that *D. viviparus* exhibited the slowest motility and migration, and the intestinal trichostrongyles of cattle and sheep, and the soil nematodes, the highest.

Concurrently with the development of traps on the dishes, the nematodes themselves were trapped, and after 12 hours very few were observed to migrate freely on the surfaces. However, at 48 hours motile *D. viviparus* larvae were seen at both inoculum levels.

Table 7 shows that, in both series, *A. oligospora* possessed a high and almost instantaneous trapping efficiency towards all nematodes, in that approximately 80% or more were caught after 30 minutes. Most species were completely trapped after six hours. One interesting exception was *R. wohlgemuthi* where a few free individuals were recorded as late as 27 hours after the experiment had started. On one occasion, a strangled and killed female was observed from which larvae were gradually released, and this may perhaps explain the persistent finding of few individuals of this species.

On close examination, it was observed that ensnared third-stage larvae of all the parasitic nematodes apart from the cyathostomes continued to wriggle in the traps for many hours, a few up to 27 hours, unlike the soil nematodes and the cyathostomes which apparently became paralyzed within a few hours after entrapment.

During the early part of the experimental period, nematodes of all categories were occasionally seen migrating freely with traps and hyphal branches attached to their cuticle, suggesting that they had succeeded in liberating themselves. However, they were apparently recaptured. On monitoring the distribution of the nematodes on the dishes, it was noticed that the nematodes on the fungus dishes were widely dispersed over the surface and in close contact with the hyphae. On the fungus-free control dishes, they tended to accumulate at the edge of the agar.

TABLE 6

Induction of loops in *A. oligospora* by various nematodes

	Number of loops per mm^2								
	0h	3h	6h	9h	12h	15h	21h	27h	48h
Series 1:									
C. oncophora	0	2	2	22	39	33	37	72	105
D. viviparus	0	0	0	0	0	0	0	0	0
D. viviparus+	0	0	0	0	0	0	0	3	10
C. curticei	0	0	0	8	13	13	29	54	52
H. contortus	0	0	0	21	27	36	33	60	61
Oe. dentatum	0	0	1	0	1	2	5	3	12
Oe. quadrispinulatum	0	0	2	3	2	1	2	7	8
N. dubius	0	0	2	0	0	2	n.d.	9	25
P. redivivus	0	0	0	27	41	89	114	138	149

Series 2:									
C. oncophora	0	0	44	176	221	199	242	284	284
O. ostertagi	0	1	53	238	202	271	281	297	317
Oe. dentatum	0	0	4	55	27	57	115	129	111
Oe. quadrispinulatum	0	0	0	28	48	50	88	108	99
Cyathostoma spp.	0	0	10	133	153	138	178	202	167
N. dubius	0	0	2	8	17	30	41	50	73
P. redivivus	0	0	44	251	217	301	329	296	340
R. wohlgemuthi	0	0	120	147	199	265	229	303	300

+ Approximately 5000 larvae per dish.

n.d.: not determined.

TABLE 7

Trapping efficiency of *A. oligospora* against various nematodes

Number of freely migrating nematodes on fungus dishes with pre-induced loops expressed as a percentage of that on fungus-free control dishes

	0h	0.5h	3h	6h	9h	12h	15h	21h	27h	48h
Series 1:										
C. oncophora	100	2	0	0	0	0	0	0	0	0
D. viviparus	100	13	0	0	0	0	0	0	0	0
D. viviparus+	100	13	1	0	0	0	0	0	0	0
C. curticei	100	4	4	5	3	0	0	0	0	0
H. contortus	100	0	0	0	0	0	0	0	0	0
Oe. dentatum	100	0	0	0	0	0	0	0	0	0
Oe. quadrispinulatum	100	0	0	0	0	0	0	0	0	0
N. dubius	100	5	4	0	0	0	0	0	0	0
P. redivivus	100	15	1	0	0	0	0	0	0	0

Series 2:										
C. oncophora	100	0	0	0	0	0	0	0	0	0
O. ostertagi	100	0	0	0	0	0	0	0	0	0
Oe. dentatum	100	0	0	0	0	0	0	0	0	0
Oe. quadrispinulatum	100	8	0	0	0	0	0	0	0	0
Cyathostoma spp.	100	3	0	0	0	0	0	0	0	0
N. dubius	100	21	17	6	3	3	0	0	0	0
P. redivivus	100	1	2	0	0	0	0	0	0	0
R. wohlgemuthi	100	2	2	0	0	0	2	0	3	0

+ Approximately 5000 larvae per dish.

EXAMPLE 10

The survival of hyphae and spores of Arthrobotrys oligospora in in vitro experiments

In vitro experiment

Spores and hyphae of *A. oligospora* were incubated at 39°C in water, pepsin-HCl and ruminal fluid in order to simulate the gastrointestinal passage of the fungal material in calves, the experiment being performed according to a modified method of J.M.A. Tilley and R.A. Terry, "A two stage technique for the in vitro digestion of forage crops", *Journal of British Grassl. Soc. 18*, 1963, pp. 104-111. Incubation times were from 0 hours (controls) to several weeks.

After the incubation, the presence of viable fungal material was determined by inoculating Petri dishes containing an agar substrate to which had been added nematodes (*Panagrellus*). The statistical MPN (most probable number) method (DeMan, J.C., "The probability of most probable numbers", *Eur. J. Appl. Microbiol. 1*, 1975, pp. 67-78) was employed for quantitative determinations.

The results are inconclusive in that they show large variations. However, some general conclusions appear from Table 8 below. It appears from the Table that hyphae have a survival rate which is comparable

to that of conidia (spores) and that, of the tested fluids, ruminal fluids seems to have the highest inhibitory effect on the survival of the fungus.

TABLE 8

	Conidia	Hyphae
Water	- 4 days	- 2 weeks
Pepsin-HCl	- 2 days	- 2 days
Ruminal fluid	- 6 hours	- 4 hours

EXAMPLE 11

The in vitro survival of Arthrobotrys oligospora spores and hyphae

The concentration of fungal material in the starting material was determined by preparing 10^{-2}, 10^{-3} and 10^{-4} dilutions of spores by mixing 1 g of hyphal material with 9 ml of sterilized tap water. Petri dishes containing CMA medium (as described in Example 1) were inoculated with 0.5 ml of fungal material per Petri dish (5 Petri dishes per dilution) and incubated at 25°C overnight. On the following day, 0.5 ml per Petri dish of a *Panagrellus* suspension was added, and the recovery of the fungal material was determined by observing any growth of the fungus after 2 weeks of incubation. During the first week, the Petri dish was kept only partly covered.

1 ml of the diluted fungal material was added to 9 ml of water, 9 ml of pepsin-HCl and 9 ml of filtered ruminal fluid in order to simulate the gastrointestinal passage of the fungal material in calves. The pepsin-HCl was prepared by dissolving 2 g of 1:10,000 pepsin (Sigma P 7000) in 850 ml of sterilized tap water, adding 100 ml of 1M HCl and making up to 1000 ml with sterilized tap water. Ruminal fluid was taken on the day when the experiment was started.

The flasks containing the spores and hyphae were incubated at 39°C in a shaking bath for the first day and then in a thermostatic cupboard. One flask was used for each incubation time. 1 ml of fungal material

added to 9 ml of water was incubated at 25°C as a control. After each incubation time, the fungal material was inoculated in Petri dishes containing *Panagrellus* as described above. Five dishes were used for each incubation time.

Incubation times:

Control: 0, 1, 2 days, 1 week.
Water: 0, 2, 4 and 8 hours, 1, 2, 3, 4 days, 1 week.
Pepsin-HCl: 0, 2, 4, 8 hours, 1, 2, 3, 4 days, 1 week.
Ruminal fluid: 0, 2, 4, 8 hours, 1, 2, 3, 4 days, 1 week.

The results are shown in Tables 9 and 10 where all inoculations were made from a 10^{-2} dilution of the fungal material.

TABLE 9

Incubation of *Arthrobotrys oligospora* at 39°C

Treatment	Time (numer of positive Petri dishes out of 5 inoculated plates) 0h	2h	4h	8h	1day	2days	3days
			Submerged mycelium (A)				
Water	5	5	5	5	5	1	0
Ruminal fluid	5	5	5	0	0	0	0
Pepsin-HCl	3	0	0	1	0	0	0
			Aerial mycelium (B)				
Water	5	5	5	5	4	5	0
Ruminal fluid	4	5	4	0	0	0	0
Pepsin-HCl	3	0	0	1	0	0	0
			Conidia (C)				
Water	5	5	5	5	5	5	0
Ruminal fluid	3	5	4	0	1	0	0
Pepsin-HCl	4	3	0	0	3	0	0

TABLE 10

Control incubations of *Arthrobotrys oligospora* at 25°C

Material	Time			
	0	1day	2days	1week
Submerged mycelium (A)	5	5	5	5
Aerial mycelium (B)	5	5	5	5
Conidia (C)	5	5	5	5

It appears that the fungal material of *A. oligospora* shows a very poor viability when incubated in ruminal fluid and Pepsin-HCl and only a somewhat higher viability when incubated in water, all incubations being negative by day 3. In contrast, control incubations at 25°C in water show that the fungal material is viable for at least a week.

EXAMPLE 12

Field experiments on the ability of Arthrobotrys oligospora to reduce the extent of infection by Ostertagia ostertagi in grazing calves

5 parasite-free donor calves were each orally infected with a monoculture of between 30,000 and 100,000 infective larvae (L_3) of a Danish strain of *Ostertagia ostertagi* Stiles (*Trichostrongylidae*). Samples of fresh faeces containing *O. ostertagi* eggs were collected daily for experimental purposes and stored for not more than 6 weeks at 5°C.

Arthrobotrys oligospora was cultivated in a liquid medium containing 42 g of liquid malt extract; 1.68 g of asparagine; 1.5 g of $CaCl_2$, 1 g of $K_2HPO_4 \cdot 7H_2O$; 0.5 g of KCl; 0.01 g of $FeSO_4$/100 ml of H_2O and 1 litre of distilled water substantially as described in Example 4. The fungus did not develop conidia in this medium. 200 ml portions of inoculated media were cultivated for between 1 and 2 weeks at 20°C in shaking bottles to ensure aeration of the medium.

Before use, the liquid medium was removed and replaced by tap water containing 0.1% Tween® 80 and the mycelia in the suspension were fragmented by placing them in an "Osterizer" blender for 2 minutes. The size of the mycelia fragments was between 30 and 300 µm. Finally the suspension was adjusted to different levels of gram mycelia per ml. For each mycelial suspension used, the number of mycelia fragments per ml was counted and the percentage of viable (germinating) mycelia fragments was measured on a corn meal agar medium (Lysek & Nordbring-Hertz, 1981 (42)). Only newly harvested and fragmented mycelia were used in the field experiments.

Laboratory experiment on the influence of mycelial concentrations on nematode entrapment in cow pats

A faecal portion from the donor calves was thoroughly mixed for 20 minutes in a cement mixer. The count of *O. ostertagi* in the faeces was found to be 700 eggs/g faeces. From this sample, ten 500 g portions were prepared. Two 500 g portions of faeces were separately inoculated with each of the following amounts of *A. oligospora* mycelia/kg faeces; 0.0 g (control), 0.03 g, 0.075 g, 0.19 g or 0.48 g. The proportion of germinating mycelia fragments in the inocula was found to be 88%.

The faecal portions, which were moulded into dome-shaped cow pats (diameter 16 cm), were placed in small plastic buckets with a bottom layer of 1 litre of parasite-free sterile soil. The cow pats were kept in the laboratory at 20°C and 60-90% RH. Three times a week each cow pat was sprinkled with 10 ml of water.

After a cultivation period of 17 days, samples of between 2 and 5 grams were taken from the edge of the cow pats, and the number of infective larvae per gram of faeces was determined by a modified Baermann technique (Jørgensen & Madsen, 1982 (45)).

Field experiment

The experimental pasture

The experiment was carried out on a parasite-free pasture which had not been grazed for several years. The soil was a brown sandy clay with a pH of 6.5 and an organic matter content of 4% of soil dry weight. Before the start of the experiment, the pasture was divided into two comparable plots of equal size (20 x 100 metres; 0.2 ha).

At regular weekly intervals (seven times in all), batches of approximately 80 kg of faeces from the donor calves were brought to the experimental pastures. The faeces was carefully mixed in a cement mixer for 20 minutes to obtain an even distribution of *O. ostertagi* eggs. From the mixed faecal portion, 10 small subsamples were taken to determine the concentration of parasite eggs by a modified McMaster method (Henriksen & Aagaard, 1976 (49)). Half of the faecal portion was then removed. The remaining portion was mixed with fragmented mycelium of *A. oligospora* in the cement mixer for another 20 minutes, giving a mycelial concentration of 0.25 g per kg of faeces when applied in a 10 ml aqueous suspension per kg of faeces. Counts were made according to Henriksen & Korsholm, 1983 (44).

259 fungus-inoculated 1 kg dome-shaped cow pats (diameter 20 cm; height 3 cm) were placed in seven long rows in one plot at intervals of 2.5 m. The same number of similarly-sized uninoculated cow pats were placed in the second plot in a similar way.

Determination of infective Ostertagia ostertagi larvae in cow pats

At weekly intervals, samples of a few grams of faeces were taken from the crust at the edge of 5 randomly selected cow pats in each row. Infective larvae were isolated by a modified Baermann technique (Jørgensen & Madsen, 1982 (45). When tracer calves were subsequently turned to grass in the two plots, they destroyed the cow pats so that no faecal samples could be taken after that time.

Determination of infective Ostertagia ostertagi larvae in herbage around the cow pats

Small herbage samples were taken 10 cm from all the cow pats at weekly intervals. After that, herbage samples were taken at biweekly intervals. The samples from each row of cow pats were combined into one sample. When tracer calves were subsequently present in the plots, however, it was impossible to find the cow pats in the rows because of disintegration. Instead, herbage samples from each field were taken 10 cm from randomly selected cow pats, including cow pats deposited by the tracer calves, and combined into one sample. The number of infective *O. ostertagi* larvae were determined by the agar technique of Jørgensen, 1975 (46), and Mwegoha & Jørgensen, 1977 (47).

Herbage cutting

In the period before the tracer calves were turned to grass, the height of the herbage was kept between 8 and 15 cm. Herbage within a distance of 20 cm from the cow pats was cut to a height of between 20 and 30 cm in order to simulate the grass tufts around cow pats which normally occur on grazed pastures.

Grazing by tracer calves

Eight 5-6 month-old parasite-free Jersey calves with body weights between 146 and 170 kg were used in the experiment. The calves were divided into two groups with approximately the same total weight and turned to grass in each of the two plots on the 15th of July 1987. Two months later, the calves were slaughtered. During the second half of the grazing period, grass was scarce and the calves were, in addition, offered parasite-free hay and straw as well as concentrates.

Examination of tracer calves

The calves were weighed at monthly intervals and rectal, faecal and blood samples were taken at biweekly intervals.

Faecal egg counts were made according to a modified McMaster technique (Henriksen & Aagaard, 1976 (49)) and faecal larval counts were made by the method described by Henriksen & Korsholm, 1983 (44).

Blood samples were analysed for serum pepsinogen and serum albumin. Serum pepsinogen was determined according to Ross et al., 1967 (50) and serum albumin was determined by an immunological method described by Mancini et al., 1965 (51).

The abomasum of each calf was analysed by sieving 1/10 of the mixed content of each abomasum through a sieve with a mesh size of 200 µm to retain adult parasites. The abomasal mucosa were removed by scraping with a knife. The isolated mucosa was digested in a pepsin/hydrochloric acid mixture (1000 ml of H_2O; 150 ml of 1N HCl, 8 g of pepsin (1:3000)) at 39°C for half an hour. The digested suspension was sieved through a fine sieve with a mesh size of 36 µm to retain larval stages of the parasite. The retained materials from the abomasal contents and the mucosa were preserved in iodine until the parasites were identified and counted.

Statistical analysis

All statistical analyses were made by Student's t-test on ln transformed data.

Meteorological observations

The weekly mean, maximum and minimum temperatures (°C) 2 m above ground level and the total weekly rainfall (mm) were recorded at a meteorological station situated 1 km from the plots.

Results

Laboratory experiment on the influence of mycelial concentrations on nematode entrapment in cow pats

The influence of increasing concentrations of *Arthrobotrys oligospora* mycelia fragments on the number of infective *Ostertagia ostertagi* larvae in cow pats is shown in Table 11. It appears from the Table that at concentrations between 10 and 180 million mycelia fragments per 1 kg of cow pats, the fungus showed a 57-71% reducing effect on *O. ostertagi* recoveries from inoculated cow pats compared with recoveries from control cow pats. The results, however, did not show any

increasing trapping efficiency with increasing mycelial concentrations.

TABLE 11

Weight (g) of mycelium per 1 kg of cow pat	Number of mycelia fragments per 1 kg (mill)	L_3PG
0	678	
0.030	10	224 (67%)
0.075	30	197 (71%)
0.190	70	259 (62%)
0.480	180	290 (57%)

Field experiment

Cow pat samples

As shown in Table 12, it was decided to use a constant concentration of 0.25 g fragmented *A. oligospora* mycelia per 1 kg of faeces in the category of cow pats placed in the fungus-inoculated plot seven times during the summer of 1987.

The seven different mycelial suspensions used contained 15-136 million mycelia fragments per 1 kg of faeces. The percentage of viable germinating fragments was found to be between 76 and 82%.

TABLE 12

Date of placing the cow pats in 1987	Weight (g) of mycelia per 1 kg of cow pat	Number of mycelia fragments per 1 kg cow pat (mill)	Percentage of viable mycelia fragments
3/6	0.25	15	76
10/6	0.25	64	80
17/6	0.25	94	78
24/6	0.25	136	80
1/7	0.25	112	82
8/7	0.25	72	82
15/7	0.25	42	77
Mean	0.25	76	79

The number of *O. ostertagi* eggs per gram of faeces (EPG) in the seven batches of faeces from donor calves is shown in Table 13. The eggs per gram faeces (EPG) value in the first faecal batch was high (415) compared with the EPG counts from the remaining 6 batches (60-200). The results presented in Table 13 also show that on average, admixture of *A. oligospora* mycelia fragments to half of the seven batches of faeces resulted in a 51% reduction in *O. ostertagi* recoveries from faecal cultures.

TABLE 13

Date of placing the cow pats in 1987	EPG[a]		L_3PG[b]			
			+ fungus		- fungus	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
3/6	415	183	34	22	229	72
10/6	140	70	N.D.		N.D.	
17/6	200	82	22	13	51	35
24/6	115	100	30	12	63	11
1/7	95	86	24	16	31	7
8/7	60	32	30	17	36	22
15/7	125	92	21	11	70	36

a) averages of 10 measurements
b) averages of 5 measurements
S.D. = standard deviation
N.D. = not determined

When the cow pat experiment was conducted (in the summer of 1987), the weather was rainy with relatively low temperatures. Under these conditions, it took *O. ostertagi* 1 to 3 weeks to develop from eggs to infective larvae. Therefore, no infective larvae were found in the rows of cow pats deposited 1 and 0 weeks before the tracer calves were turned to grass.

Generally, fewer infective *O. ostertagi* larvae developed in inoculated cow pats than in control cow pats. The total number of infective *O. ostertagi* larvae measured in all rows of the inoculated cow pats in the period until the tracer calves were turned to grass was subject to a 42% reduction compared with the total number of infective larvae measured in all rows of the uninoculated control cow pats during the same period.

Herbage samples

The rows of cow pats placed at the beginning of the experiment lead to increasing herbage infectivity about four weeks later. Most likely, the transmission of infective *O. ostertagi* larvae from cow pats

to herbage was facilitated by frequent rain about four weeks after the cow pats had been placed.

The herbage infectivity around the rows of cow pats placed over the next six weeks from the six subsequent batches conformed to a common pattern since a large proportion of the infective *O. ostertagi* larvae spread to the herbage during the same period. No doubt the increase in herbage infectivity was triggered by a prolonged heavy shower which gave 69 mm of rain over two days just after the tracer calves had been turned to grass.

The herbage samples collected during the last month of the experiment were taken from randomly selected cow pats including cow pats deposited by the tracer calves. During this period, both groups of calves were passing *O. ostertagi* eggs in their faeces and it is assumed that the calves were responsible for a recontamination of the fields. This assumption was confirmed by the rise in herbage infectivity on both fields during the last month.

In general, fewer infective *O. ostertagi* larvae spread to the surrounding herbage in the fungus-inoculated plot than in the control plot. The total number of infective *O. ostertagi* larvae measured in the herbage around cow pats in all rows in the inoculated plots was reduced by 50% compared with the total number of infective larvae measured around cow pats in all rows in the control plot over the first 8 weeks of the experiment. During the last month when both fields were recontaminated, the predatory fungus *A. oligospora* was responsible for a 71% reduction in total herbage infectivity.

Tracer calves

The results of the tracer calf experiment are presented in Table 14.

TABLE 14

Analysis of calves	Plot +/- fungus	Dates and number of weeks after turning the tracer calves to grass in 1987					
			15/7 week o	29/7 week 2	12/8 week 4	26/8 week 6	9/9 week 8
Body weight (kg)	+	Mean	16o		171		163
		S.D.	1o		5		1o
	-	Mean	158		163		147
		S.D.	1o		15		11 *
Number of parasite eggs per gram of faeces(EPG)	+	Mean	o	o	75	125	7o8
		S.D.	o	o	32	67	733
	-	Mean	o	o	296	292	658
		S.D.	o	o	117 ***	217	182
Number of infective parasite larvae per gram of faeces (L_3PG)	+	Mean	o	o	3o	87	N.D
		S.D.	o	o	19	47	
	-	Mean	o	o	99	187	N.D.
		S.D.	o	o	39 **	157	
Serum pepsinogen in blood(tyrosine units per litre)	+	Mean	o.43	o.75	1.o8	1.85	2.68
		S.D.	o.o5	o.4o	o.55	o.74	1.o9
	-	Mean	o.38	o.5o	1.oo	3.15	4.o3
		S.D.	o.o5	o.16	o.91	o.72 *	o.69
Albumin in blood (gram per litre)	+	Mean	33.8	34.o	34.o	32.o	29.8
		S.D.	2.2	1.8	1.4	2.6	1.5
	-	Mean	34.o	34.3	32.8	32.o	29.5
		S.D.	1.4	1.3	3.3	1.8	2.1
Number of adult parasites in the abomasum	+	Mean					44.378
		S.D.					18.934
	-	Mean					77.o45
		S.D.					13.49o **
Number of larval parasites in the abomasal mucosa	+	Mean					9.918
		S.D.					4.718
	-	Mean					8.475
		S.D.					5.694

Four calves grazed in a plot with cow pats inoculated with fragmented mycelia of the fungus *Arthrobotrys oligospora* (+) while another four calves grazed in a plot with fungus-free control cow pats (-). S.D.: standard deviation. N.D.: not determined. Asterisks indicate significant differences between the two groups of calves using a Student's t-test.

* $p<0.10$, ** $p<0.05$, *** $p<0.01$.

It appears from the Table that after 4 weeks the average body weight gain for the calves grazing in the inoculated plot was 11 kg compared with only 5 kg for the control group (Table 14). 4-8 weeks after the calves had been turned to grass, grass was scarce and both groups of calves were forced to graze close to the cow pats where the herbage infectivity reached high levels during the same period. Most likely, the high uptake of infective *O. ostertagi* larvae in the period from week 4 to 8 was responsible for an average body weight loss of 8 kg for calves grazing in the inoculated plot and 16 kg for calves grazing in the control plot in that period. When slaughtered the group of calves grazing in the control plot had an average body weight which was 16 kg less than the group of calves grazing in the inoculated field. This difference was statistically significant.

Eggs of *O. ostertagi* were detected in faecal samples from both groups of calves from week 4 onwards. At weeks 4 and 6, the average EPG values for the group of calves grazing in the inoculated plot were less than half the average values for the group of calves grazing in the control plot.

Four weeks after the tracer calves had been turned to grass, this difference was statistically significant. However, at the end of the experiment there was no difference in the average egg counts between the two groups.

The number of infective *O. ostertagi* larvae that developed in faecal cultures (L_3PG) confirmed the course of the EPG values in the two groups of calves.

Analysis of blood samples taken 6 and 8 weeks after turning to grass showed that the average concentrations of serum pepsinogen in the

calves grazing in the inoculated plot were subject to a 41 and 33% reduction, respectively, compared with the group of calves in the control plot. These differences were statistically significant. The serum pepsinogen concentrations in both groups of calves rose to severe levels in the last part of the grazing period, which indicated that both plots were heavily infested with *O. ostertagi*.

During the last 4 weeks on grass, there was a drop in albumin concentration in the blood of both groups of calves, but there was no difference in albumin concentrations between the groups.

After slaughtering, the abomasal mucosa in both groups of calves showed all the typical signs of clinical ostertagiasis. As appears from Table 14, there was no difference in the fraction of larvae found in the mucosa of the two groups of calves. However, the number of adult parasites found in the abomasa of the group of calves grazing in the fungus-inoculated plot was reduced by 42% compared with the number found in the control group. The difference was statistically significant. All tracer calves harboured a pure strain of *O. ostertagi*.

The results from the tracer calf experiment showed that calves grazing in the plot containing fungus-inoculated cow pats had a significantly lower parasite burden and a 16 kg higher body weight than calves grazing in the control plot. Although both groups of calves developed clinical ostertagiasis, the predatory fungus *A. oligospora* in this way clearly demonstrated a beneficial effect.

EXAMPLE 13

Titration of Arthrobotrys oligospora in horse faeces

CMA plates were inoculated with *A. oligospora* and incubated as described in Example 1. After 20 days, the plates were washed with 10 ml of sterile water and the water containing the fungal material was used to inoculate shake flasks containing 100 ml of a nutrient medium (YPG) containing 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1.5% glucose.

The flasks were incubated in the dark at 25°C on a shaking apparatus for 4 days. The fungal culture was then blended for 30 seconds at the highest speed in a Waring blender.

The culture medium was filtered off *in vacuo* through a Whatman filter.

The harvested mycelium was suspended in isotonic saline (2.30 g of mycelium in 40 ml). The suspension was adjusted to the following concentrations per 5 ml corresponding to the dosage per 500 g of horse faeces:

0.100 g of mycelium - 2.4×10^7 mycelia fragments[x)]
0.075 g of mycelium - 1.8×10^7 mycelia fragments
0.050 g of mycelium - 1.2×10^7 mycelia fragments
0.025 g of mycelium - 6×10^7 mycelia fragments

x) The mycelia fragments were counted in a Fuchs-Rosental hemocytometer.

A. oligospora conidia were scraped from sporulating colonies, grown on YPG medium supplemented with 2% Difco Bacto agar and incubated at 25°C for 14 days in 12 hours of light/12 hours of darkness.

The conidia were suspended in isotonic saline, and the following dilutions were prepared:

10^7 conidia per 5 ml
10^6 conidia per 5 ml
10^5 conidia per 5 ml
10^4 conidia per 5 ml.

The germinating property of the conidia was 100% within 6 hours at 25°C which was tested on cellophane strips placed on 2% aqueous agar. Strips containing conidia were embedded in lactophenol cotton blue on slides and the percentage of germination was determined under a light microscope.

10 kg of horse faeces were mixed thoroughly with horse strongylide eggs. The faeces contained 1170 horse strongylide eggs per gram (96%

Cyathostoma spp. and 4% *Strongylus* spp.). 500 g portions were then measured out and mixed thoroughly with a 5 ml aqueous suspension of mycelium and conidia, respectively, at increasing concentrations. Control faeces was admixed with 5 ml of pure water. Two determinations were made at each concentration of the fungal material by placing 500 g portions in plastic buckets (diameter 25 cm, height 25 cm) provided with 1 litre of autoclaved vegetable mould. Each bucket was covered with a lid provided with air holes and left to stand at room temperature. The faeces was kept moist by spraying each portion with 25 ml of water a day.

After 15 and 30 days of incubation, 5 g samples were taken from each portion of faeces. Infective parasite larvae (L_3) were isolated from faeces by a modified Baermann technique (Jørgensen & Madsen, 1982 (45)). The number of L_3-larvae was then quantified by the method described by Henriksen and Korsholm, 1983 (44).

The results appear from Tables 15 and 16 in which the reduction of the number of infectious larvae compared to the control is given as a percentage in parentheses.

TABLE 15

	MYCELIUM				CONIDIA		
		L_3/g			L_3/g		
g mycelium per kg of faeces	A	B	X	Number of conidia per g of faeces	A	B	X
0.00	12234	5438	8836	0	6052	3317	4685
0.05	648	1133	891 (90)	20	2330	3010	2670 (43)
0.10	5136	4760	4948 (44)	200	2869	3117	2993 (36)
0.15	1108	1804	1456 (84)	2000	1339	2558	1949 (58)
0.20	2806	549	1678 (81)	20000	760	1430	1095 (69)

TABLE 16

g mycelium per kg of faeces	MYCELIUM L_3/g A	B	X	Number of conidia per g of faeces	CONIDIA L_3/g A	B	X
0.00	10313	3069	6691	0	8800	9905	9353
0.05	3189	1370	2280 (66)	20	6114	2773	4444 (52)
0.10	5783	1157	3470 (48)	200	6981	5827	6404 (32)
0.15	2164	2818	2491 (63)	2000	883	4609	2746 (71)
0.20	2944	424	1684 (75)	20000	883	971	927 (90)

EXAMPLE 14

The survival of protected Arthrobotrys oligospora material in in vitro experiments

In order to establish whether seeds may serve as a carrier for A. *oligospora*, 200 g portions of poppy seeds and sesame seeds were autoclaved in beakers containing 75 ml of deionized water. The beakers were then inoculated with 10 ml of a 3-day-old submerged culture of A. *oligospora* (cf. Example 4) grown on YPG medium containing 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1.5% glucose at 180 rpm and 23-25°C.

The inoculated seeds were covered with metal foil and incubated at 25±0.5°C for 21 days and were then air-dried at 20-25°C for 18 hours before coating and testing.

The seeds were then coated with a coating material containing 44% Eudragit® RS 30 D (from Röhm Pharma GmbH, Federal Republic of Germany; a copolymer of acrylic and methacrylic acid esters with a low content

of quaternary ammonium groups), 3% talc, 3% triethylcitrate, 50% water (w/w) and then with a coating material composed of 52.2% Eudragit® L 30 D (from Röhm Pharma GmbH, Federal Republic of Germany; an anionic polymer based on polymethacrylic acid and acrylic acid esters), 1.7% talc, 1.7% triethylcitrate and 44.4% water.

The respective coating materials were sprayed onto the seeds in a Uniglatt fluidized bed provided with a two-fluid nozzle. The coating materials which were stirred during the entire spraying process were fed to the fluidized bed through a peristaltic pump at 10-12 ml/minute. The pressure was 1 bar and the outlet air temperature 30°C.

The amount of dry coating applied on the seeds was 3 mg/cm^2 of Eudragit® RS 30 D and 2 mg/cm^2 of Eudragit® L 30 D.

To determine the degree of colonization of the seeds, i.e. the degree to which *A. oligospora* grew into the seeds, uncoated seeds were surface sterilized for 15 seconds in 70% ethanol and 90 seconds in 2% sodium hypochlorite before incubation on YPG agar (2%).

The tolerance of uncoated and coated *A. oligospora* colonized seeds to simulated gastric fluid (0.1 M HCl, 0.2% pepsin (Sigma) in water) was tested in order to determine whether the viability of the fungal material when passing through the gastrointestinal tract of an animal could be improved by providing the colonized seeds with a coating. The test was conducted in a Pharma Test (a laboratory stomach simulator device), type PTW, at 50 rpm and 38-40°C for 2 hours at pH 1.04 and 1 hour at pH 6.5 (deionized water adjusted with NaOH). After the test, 50-65 poppy and sesame seeds, respectively, were incubated on YPG agar at 23±0.5°C for a maximum of 72 hours.

The results are shown in Table 17 below in which the viability of *A. oligospora* is given as the percentage of seeds from which the fungus germinates and establishes itself on the agar plate.

TABLE 17

Type of seed colonized by *A. oligospora*	Treatment	No. of incubated seeds	No. of seeds containing viable *A. oligospora*	% seeds containing viable *A. oligospora*	Corrected % x)
Uncoated					
Poppy	Untreated control	50	37	74	
Poppy	Surface-sterilized	50	16	32	43
Poppy	Pepsin-HCl	50	2	4	5
Sesame	Untreated control	50	48	96	
Sesame	Surface-sterilized	50	12	24	25
Sesame	Pepsin-HCl	50	2	4	4
Coated					
Poppy	Pepsin-HCl	65	16	25	33
Sesame	Pepsin-HCl	64	31	48	50

x) corrected for percentage of germinating fungi from untreated control seeds rounded off to the nearest whole number.

It appears from the results of the surface sterilization treatment that the fungus has mainly colonized the outer parts of the seed.

11-12% of the well-colonized seeds have had a protective effect on the fungus.

It further appears from the results that if the colonized seeds are coated, an approximately 9 times higher viability of the fungal material is obtained after treatment with pepsin-HCl. The higher viability of coated seeds compared to surface-sterilized seeds indicate that the coating to some extent protects the hyphae established on the surface of the seeds.

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CLAIMS

1. A method of reducing the number of infective, animal parasitic nematodes in an environment of animals so as to reduce the transmission of nematode infection to animals inhabiting said environment, the method comprising administering to an animal a composition comprising a fungal material of a nematode-destroying fungus in order to provide an adequate nematode-controlling amount of the fungal material in the animal faeces, the composition being formulated in such a way that an adequate nematode-controlling proportion of the fungal material remains viable after passage through the gastrointestinal tract of the animal to which the composition is administered.

2. A method according to claim 1, in which the nematode-destroying fungus is a predatory fungus, an endoparasitic fungus or an ovoparasitic fungus.

3. A method according to claim 2, in which the fungus is a predatory fungus belonging to an *Arthrobotrys* spp. or a *Dactylaria* spp.

4. A method according to claim 3, in which the predatory fungus is *Arthrobotrys oligospora*, *Arthrobotrys tortor*, *Arthrobotrys musiformis*, *Arthrobotrys conoides*, *Arthrobothrys superba*, *Arthrobotrys arthrobotryoides*, *Dactylaria candida* or a mixture thereof.

5. A method according to claim 1, in which the animals are domestic animals.

6. A method according to claim 5, in which the animals are young animals.

7. A method according to claim 5 or 6, in which the animals to which the composition is administered are untethered animals.

8. A method according to claim 5, in which the animals are pigs.

9. A method according to claim 8, in which the composition is administered at least once every two days, preferably at least once a day, for at least 1 month, preferably for at least 2 months, during a

period where contamination of the environment by nematodes is critical.

10. A method according to claim 8, in which the environment inhabited by the animals is a pigsty.

11. A method according to claim 5, in which the animals are domestic fowl.

12. A method according to claim 11, in which the composition is administered at least once every two days, preferably at least once a day, for at least 1 month, preferably for at least 2 months, during a period when contamination of the environment by nematodes is critical.

13. A method according to claim 5, in which the animals are carnivores.

14. A method according to claim 13, in which the animals are fur animals such as mink or fox, or pets such as dogs or cats.

15. A method according to claim 5, in which the animals are herbivores.

16. A method according to claim 15, in which the animals are horses.

17. A method according to claim 16, in which the composition is administered at least once every two days, preferably at least once a day, for at least 1 month, preferably for at least 2 months, during a period when contamination of the environment by nematodes is critical.

18. A method according to claim 16, in which the environment inhabited by the animals is a stable or a pasture.

19. A method according to claim 15, in which the animals are ruminants.

20. A method according to claim 19, in which the animals are cattle, sheep, deer or goats.

21. A method according to claim 19, in which the environment inhabited by the animals is a pasture.

22. A method according to claim 19, in which the composition is administered to the ruminants at least once at a time of the year when contamination of the environment by nematodes is critical.

23. A method according to claim 22, in which the composition is administered to the ruminants at least once at a time of the year when the animals are turned to grass.

24. A method according to claim 22, in which the composition is administered at least twice during a period of at least 1 month.

25. A method according to claim 1, in which the composition is formulated so as to provide a controlled release of the fungal material.

26. A method according to claim 25, in which the composition is in the form of a bolus, matrix tablet, coated tablet or capsule, or coated granules.

27. A composition for controlling animal parasitic nematodes which comprises a fungal material of a nematode-destroying fungus and an excipient which significantly improves the viability of the fungal material in the gastrointestinal tract of an animal to which the composition is administered.

28. A composition according to claim 27, in which the nematode-destroying fungus is a predatory fungus.

29. A composition according to claim 28, in which the fungus is a predatory fungus belonging to an *Arthrobotrys* spp. or a *Dactylaria* spp.

30. A composition according to claim 29, in which the predatory fungus is selected from the group consisting of *Arthrobotrys oligospora*, *Arthrobotrys tortor*, *Arthrobotrys musiformis*, *Arthrobotrys conoides*, *Arthrobothrys superba*, *Arthrobotrys arthrobotryoides*, *Dactylaria candida* or a mixture thereof.

31. A composition according to claim 27, which comprises coated granules containing the fungal material.

32. A composition according to claim 31, in which the granules are coated with one or more coating layers of an enteric coating, a water-permeable coating or a coating which is enzymatically degradable in the abomasum, small intestine, colon or faeces.

33. A composition according to claim 33, in which the enteric coating is a coating which is soluble at the pH prevailing in the colon or faeces.

34. A composition according to claim 32, in which the enteric coating is selected from shellac, cellulose acetate esters such as cellulose acetate phthalate, hydroxypropyl methyl cellulose esters such as hydroxypropyl methyl cellulose phthalate, polyvinyl acetate esters such as polyvinyl acetate phthalate, and polymers of methacrylic acid and (meth)acrylic acid esters.

35. A composition according to claim 32, in which the water-permeable coating is selected from ethyl cellulose, cellulose acetate, cellulose propionate, cellulose butyrate, cellulose valerate, polyvinyl acetate, polyvinyl formal, polyvinyl butyral, polymethyl methacrylate, polycarbonate, polystyrene, polyester, polybutadiene, polyvinyl chloride, microporous polycarbonates, microporous polyamides, acrylic copolymers and polyurethanes.

36. A composition according to claim 27, which is in the form of a bolus.

37. A composition according to claim 36, in which coated granules containing the fungal material are embedded in a matrix.

38. A composition according to claim 16 or 17, in which the matrix material is selected from a natural or synthetic wax, a plastic, a polymer, a polysaccharide, such as an alginate, a dextrin, a starch, cellulose or a derivative thereof or agarose, a resin, a fatty alcohol, fatty acid and esters thereof, a mineral such as silica or a silicate, kaolin, bentonite, diatomaceous earth, vermiculite, pumice or

mineral wool, and a vegetable material such as wheat bran or seeds, e.g. poppy or sesame seeds.

39. A composition according to claim 18, in which the bolus matrix additionally contains an agent imparting an increased specific gravity to the composition, e.g. barium sulphate, titanium oxide, a zinc oxide or an iron salt.

40. A composition according to claim 36, in which the amount of active fungal material is in the range of 1-15 g, in particular 2-10 g per bolus.

41. A composition according to claim 27, which is in the form of a feed block containing embedded coated granules comprising the fungal material.

42. A composition according to claim 41, in which the feed block comprises molasses.

43. A composition according to claim 27, in which the composition further comprises a chemical antiparasitic agent, such as an anthelmintic, or another active agent such as a growth promoting agent, hormone, vitamin, macro- or micronutrient or amino acids.

44. A process for producing a fungal material of a nematode-destroying fungus, which process comprises

a) inoculating a suitable liquid medium with mycelium or spores of the fungus,

b) growing the fungus in submerged culture under aeration and agitation to produce a fungal material, and

c) harvesting the fungal material.

44. A process according to claim 44, in which the nematode-destroying fungus is a predatory fungus, an endoparasitic fungus or a ovoparasitic fungus.

46. A process according to claim 45, in which the fungus is a predatory fungus belonging to an *Arthrobotrys* spp. or a *Dactylaria* spp.

47. A process according to claim 46, in which the predatory fungus is selected from the group consisting of *Arthrobotrys oligospora*, *Arthrobotrys tortor*, *Arthrobotrys musiformis*, *Arthrobotrys conoides*, *Arthrobothrys superba*, *Arthrobotrys arthrobotryoides*, *Dactylaria candida* or a mixture thereof.

48. A process according to claim 44, in which the medium comprises a vegetable organic or inorganic solid support to which the fungal material adheres and on and/or in which it grows.

49. A process according to claim 48, in which the solid support comprises a spongy or porous material such as seeds, e.g. poppy or sesame seeds, or an organic or inorganic polymer such as porous polyacrylic or glass beads.

50. A method of reducing the number of infectious nematodes in the environment of animals so as to reduce the occurrence of nematode infections in animals inhabiting said environment, the method comprising administering to an animal a composition comprising a fungal material produced by the process according to any of claims 44-47 in order to provide an adequate nematode-controlling amount of the fungal material in the animal faeces.

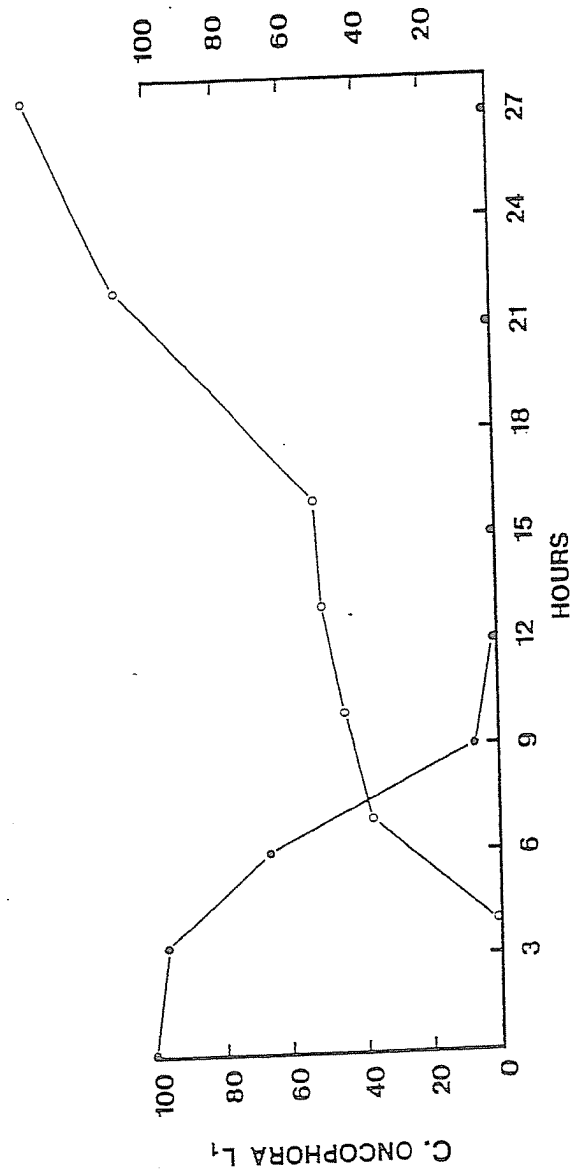

Fig.1a

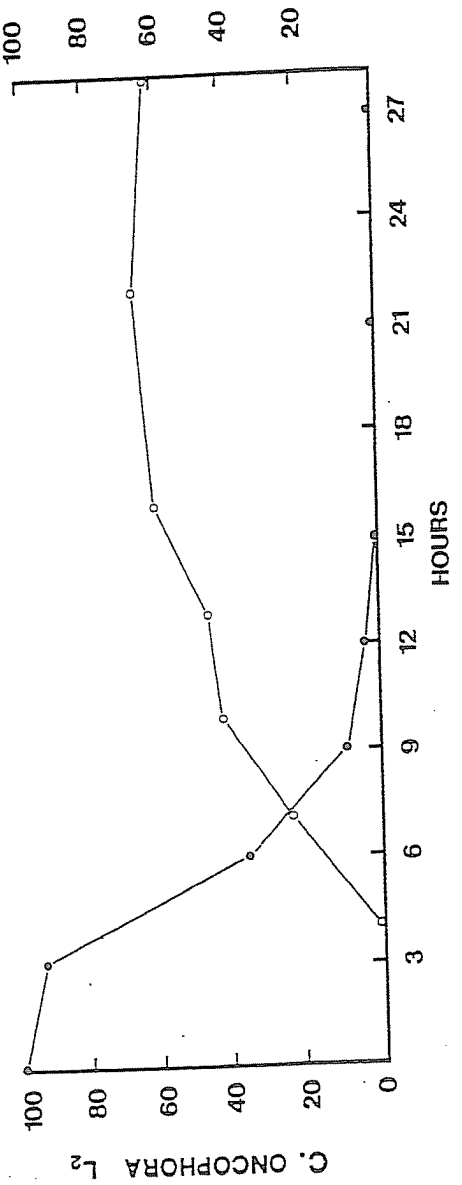

Fig. 1b

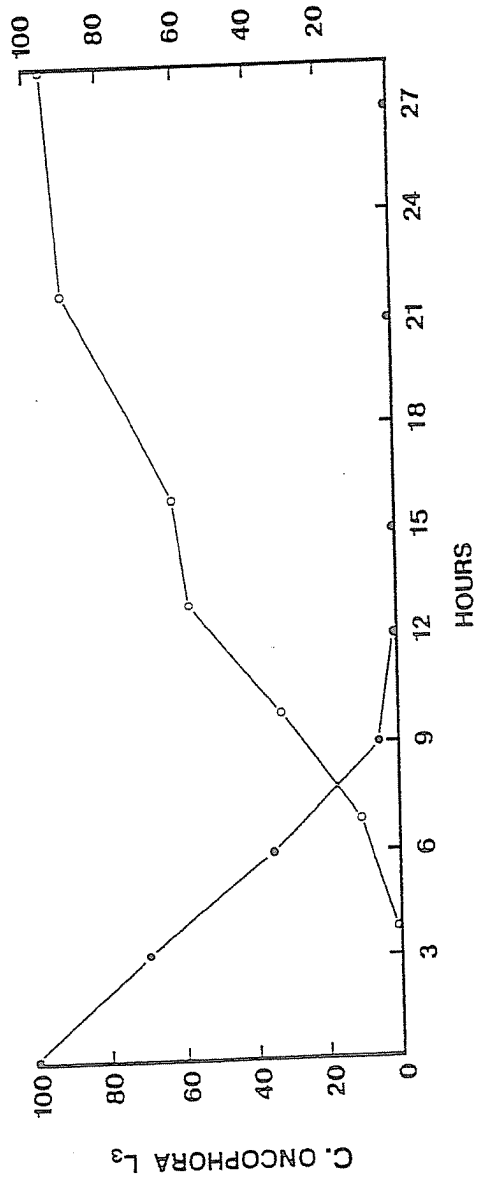

Fig. 1c

SUBSTITUTE

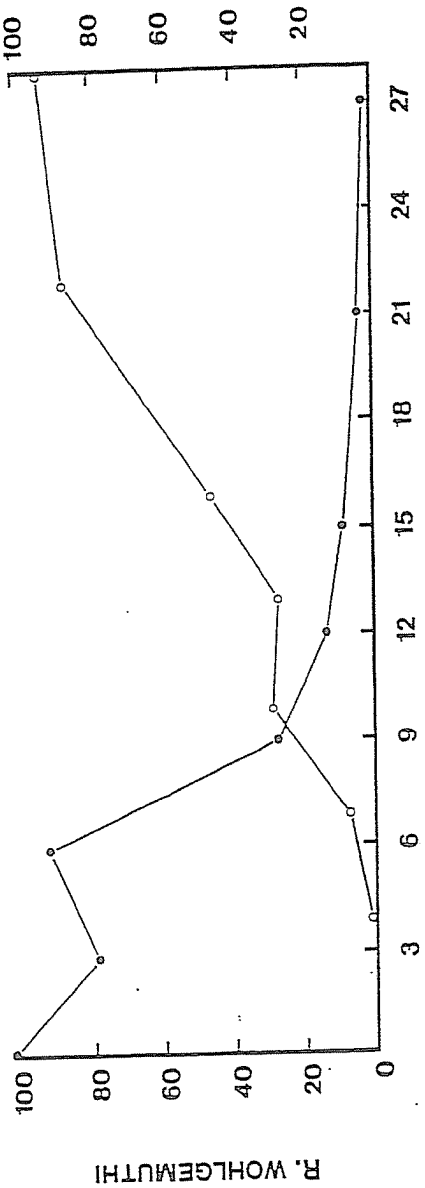

Fig. 1d

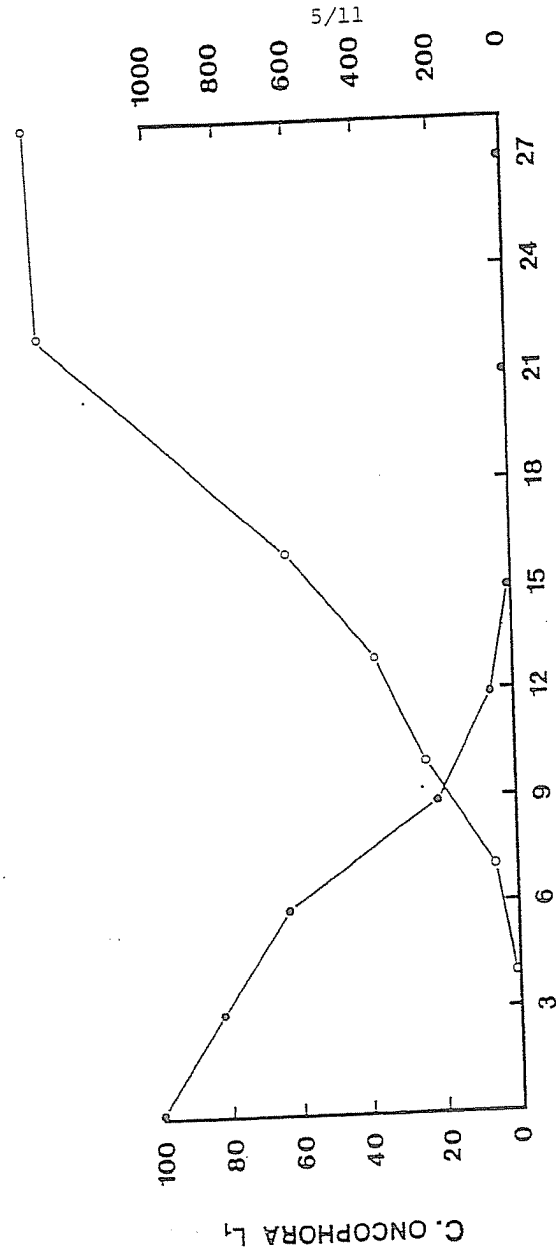

Fig. 2a

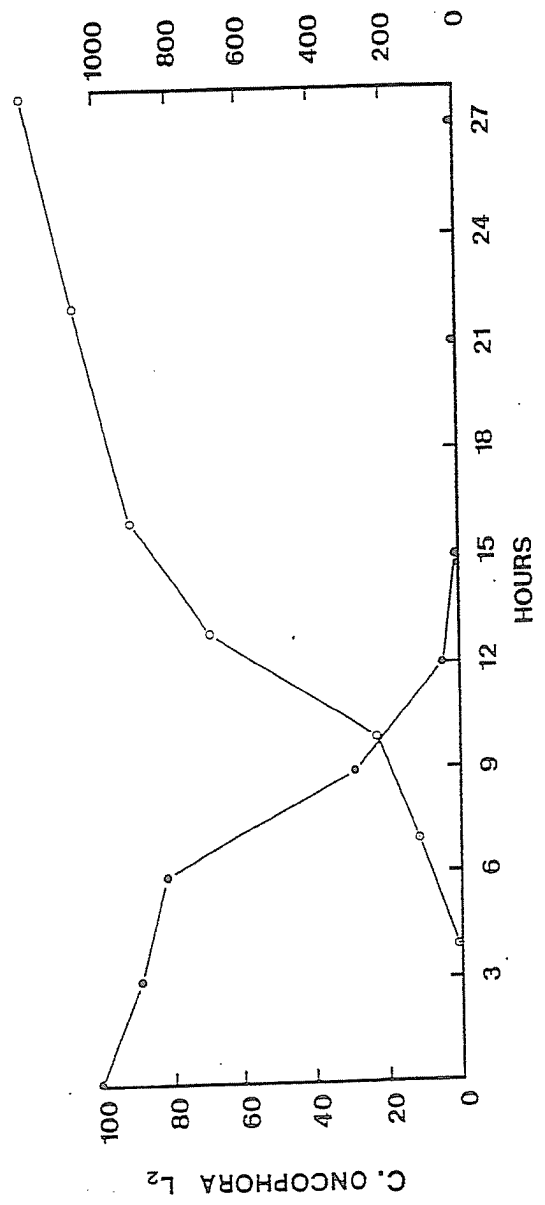

Fig. 2b

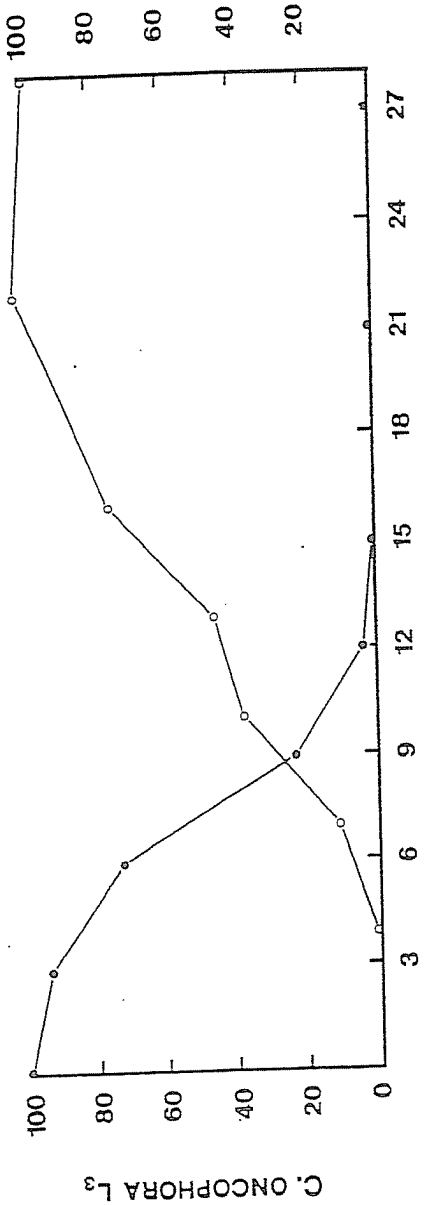

Fig. 2c

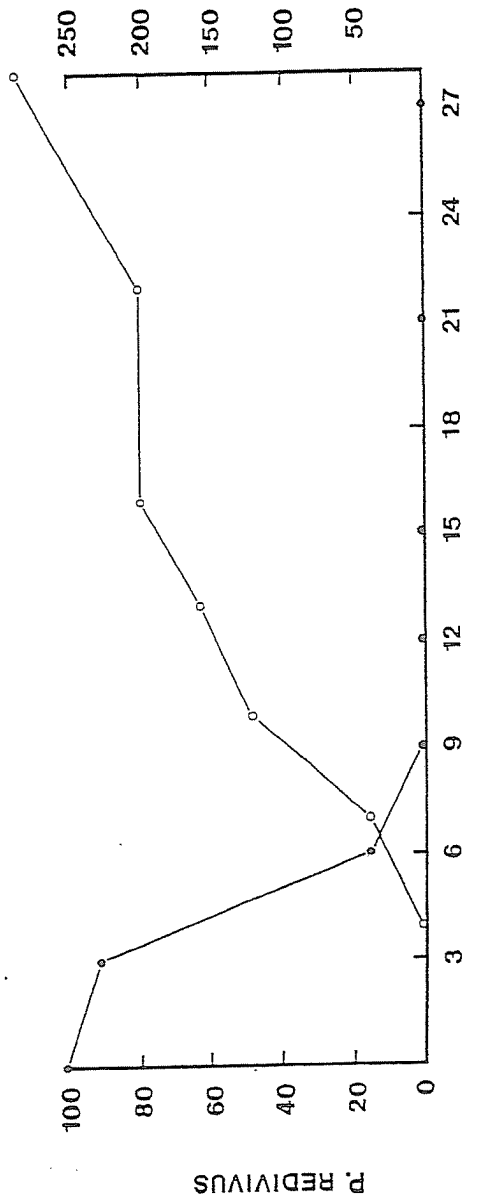

Fig. 2d

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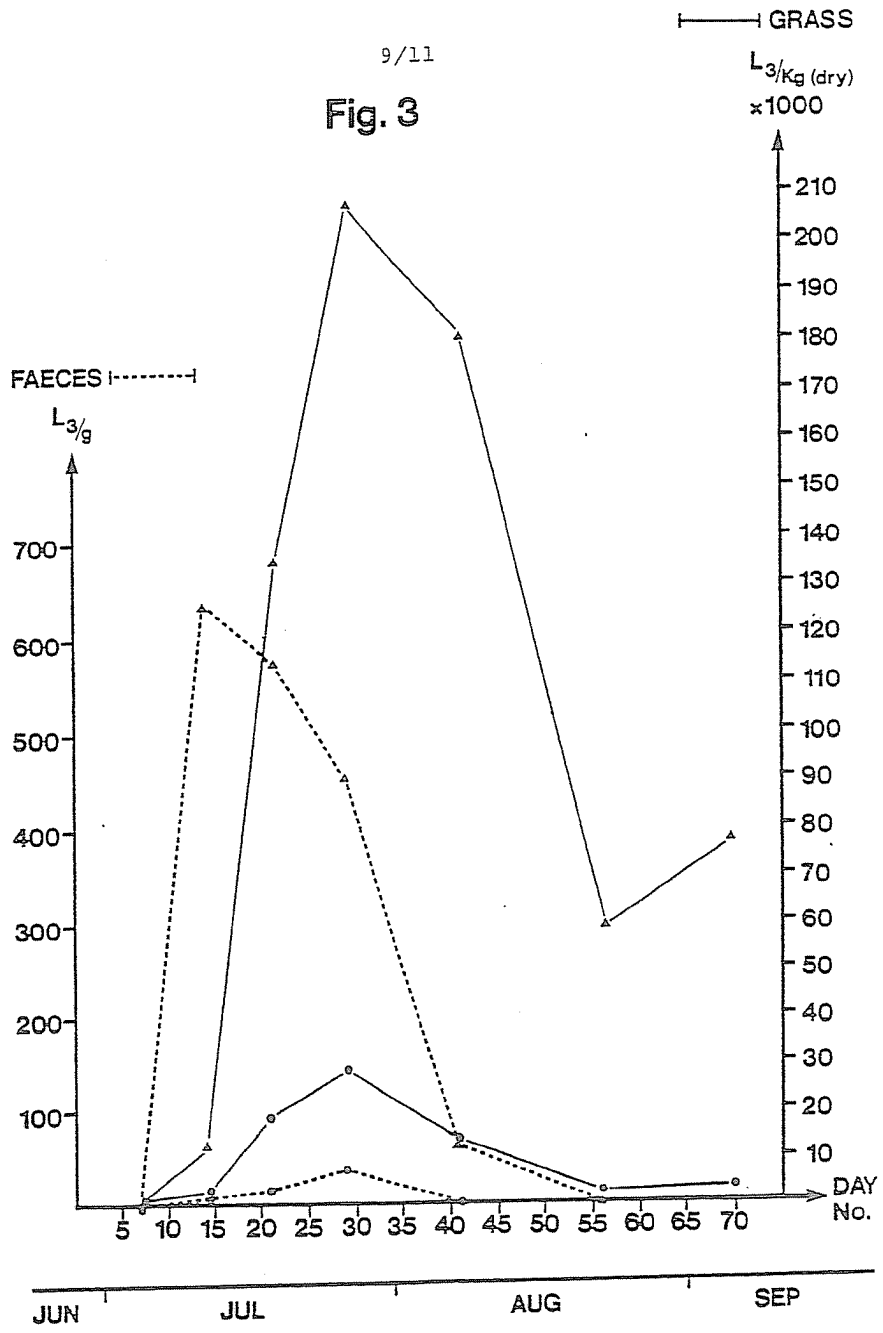
Fig. 3



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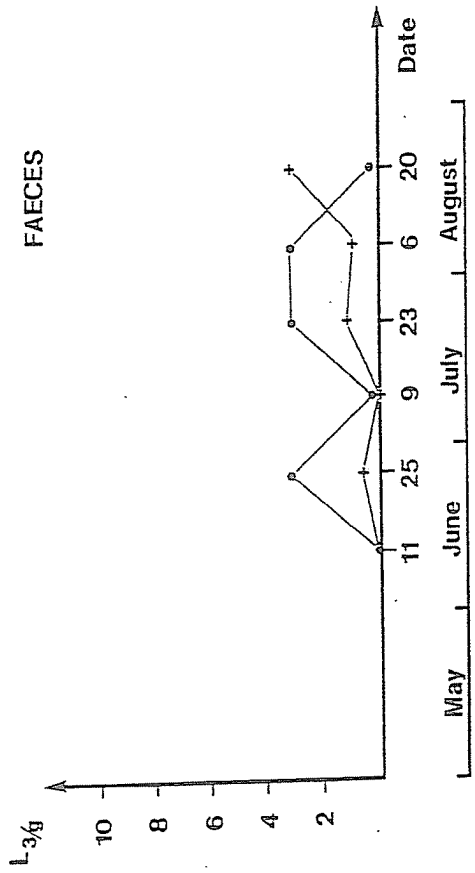

Fig. 4

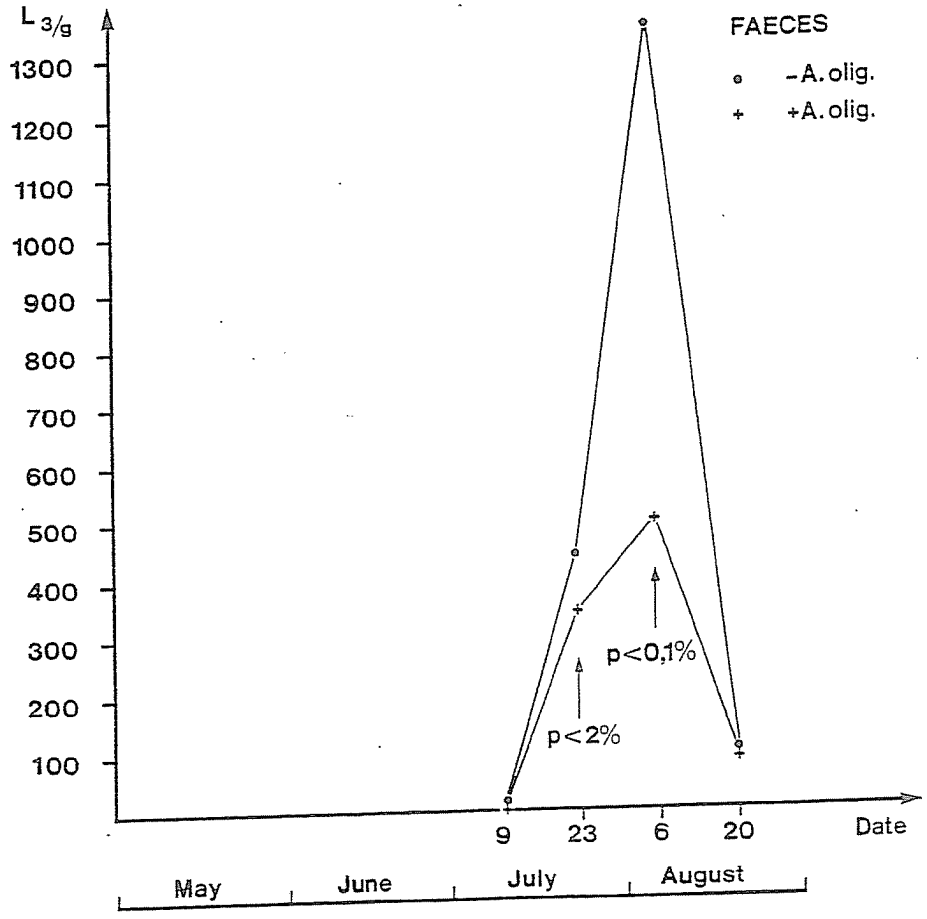

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00039

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

A 01 N 63/04

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	A 01 N 63/04, /00, /02
US Cl	424:93

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Dialog (CAB ABSTRACTS), accession numbers 0560760, Gel´minty Zhivotnykh v ekosystemakh Kazakhstana Edited by E.V.A. Gvozdev, 1985, 186-201 (Russ)	1-50
X	0526853, Izvestiya Akademii Nauk Kazakhskoi SSR, Biologicheskaya, 1986 (No. 1), 30-33 (Russ)	1-50
X	0364599, Zhivotnyi mir Kazakhstana i problemy ego okhrany, 1982, 149-151 (Russ)	1-50
X	Journal of Helminthology, 59 (1985), J. Grønvold et al, "Laboratory experiments to evaluate the ability of Arthrobotrys oligospora to destroy infective larvae of Cooperia species, and to investigate the effects of physical factors on the growth of the fungus", pages 119-125	1-50

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1988-05-17	1988 -08- 05
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Gerd Wrenne

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Proceedings, Helminthological society of Washington, 53(1986), No. 2, Washington, P. Nansen et al, "Predacious Activity of the Nematode-destroying Fungus, Arthrobotrys oligospora, on Preparasitic Larvae of Cooperia oncophora and on Soil Nematodes", pages 237-243	1-50
X	Phytoma - Défence des cultures, No. 349 (1983), J.C. Ceyrol et al, "Spécificité d'action de champignons hyphomycètes prédateurs de nématodes: conséquences pratique en lutte biologique", pages 7-9	1-50
P,X	Journal of Helminthology, 61(1987), No. 1, March, J. Grønvold et al, "Field experiments on the ability of Arthrobotrys oligospora (Hyphomycetales) to reduce the number of larvae of Cooperia oncophora (Trichostrongylidae) in cow pats and surrounding grass" pages 65-71	1-50